INTERLEUKIN-6 *TRANS*-SIGNALING: IMPLICATIONS FOR NEUROINFLAMMATION AND COGNITIVE DEFICITS IN THE AGED

BY

MICHAEL DONAHUE BURTON JR

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Animal Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Doctoral Committee:

Professor Rodney Johnson, Chair and Director of Research
Professor Gregory Freund
Professor Jeffrey Woods
Assistant Professor Ryan Dilger

Abstract

In the event of peripheral infection, aged mice exhibit a heightened central inflammatory cytokine response, as well as prolonged behavioral and cognitive deficits when compared to adults. This is attributed to a population of activated or “primed” microglia that produce excessive amounts of pro-inflammatory cytokines, such as interleukin (IL)-6. The over production of IL-6 during aging is correlated with acute behavioral deficits in response to a peripheral infection or chronic behavioral deficits in the development of neurodegenerative diseases, such as Alzheimer’s and Multiple Sclerosis . However IL-6 is implicated in pro- and anti-inflammatory processes, so it is vital to understand it’s mechanisms in aging, neuroinflammation, and neurodegeneration.

In initial studies using a microglia cell line (BV.2), we found that the IL-6 *trans*-signaling pathway stimulated MHC-II, a marker of activation. While microglia isolated from aged IL-6 knock-out animals displayed significantly less MHC-II expression. Additionally, IL-6 knock-out animals were refractory to LPS-induced sickness behavior implicating the involvement of IL-6 and microglial activation in aging. However, which arm of IL-6 signaling had the most influential effects remained unclear. Recently, IL-6 *trans*-signaling has been shown to induce the pro-inflammatory actions of IL-6 and selective blockade of the *trans*-signaling cascade using soluble gp130 (sgp130) has reduced the deleterious effects of peripheral inflammatory conditions such as Crohn’s disease and arthritis.

Although sgp130 has been shown to inhibit peripheral inflammation, its effects on the inflammatory response in the brain are unknown. If sgp130 reduces inflammation in the brain, it is rational to hypothesize that it will protect aged animals from infection-related exaggerated levels of IL-6 and behavioral deficits. In a series of studies testing this hypothesis, sgp130 was found to inhibit LPS-induced IL-6 production via STAT3-dependent action in BV.2 cells. In subsequent studies, adult and aged mice were co-administered vehicle or sgp130 via intracerebroventricular (icv) and i.p. with saline or LPS. As expected, aged mice displayed deficits in locomotor activity and hippocampal-dependent memory tasks, indicating they were more sensitive to peripheral immune stimulation. Importantly, LPS-induced sickness behavior and cognitive deficits in aged animals were mitigated by icv sgp130. These behavioral effects were mirrored by a reduction of STAT3 phosphorylation, IL-6 mRNA expression, and protein production in hippocampal tissue. Additionally, sgp130 also attenuated the induction of IL-6 protein from isolated microglia and astrocytes from the aged. These data show that inhibiting the IL-6 *trans*-signal, suggests a possible role in attenuating acute cognitive and behavioral disorders in older individuals with an infection.

Table of Contents

Chapter

Page

List of figures

List of Tables

Chapter 1

General introduction and justification

The average life expectancy in the United States has improved by almost 30 years in the last half century, a trend which can be attributed to monumental advancements in modern medicine. These advances were vaccines and remedies to infectious diseases that predominately affected young children, providing them with sufficient immunity and the opportunity to survive until adulthood. These events have generated a population shift in society where there are an increased number of middle-aged and advanced-aged individuals. It is important to note that with this increase in aged individuals, a new set of diseases, where inflammation is a major pre-disposing factor have risen to prominence.

Although vaccines were created to help our immune system mount a concerted response to infectious disease, the innate immune system has maintained its primal tenacity to respond to foreign agents and inflammation is a result. Inflammation is a response initiated by the innate immune system of an organism which releases inflammatory mediators in a protective attempt to remove injury, pathogens or stimulate healing.

With the immune response unaltered in its reactivity to foreign invaders over time, an age-dependent upregulation of the inflammatory response due to chronic antigenic stress throughout life becomes apparent. The inflammatory response takes a toll on the human body and becomes the prevalent mechanism in age-associated disease (Candore et al., 2003; Licastro et al., 2005; Mariani et al., 2003; Vasto and

Caruso, 2005). Through an immune-to-brain axis, peripheral immune infection impairs cognition and reduces self-care behaviors in the aged contributing to a vicious cycle of higher hospitalization rates and a sizeable cost of healthcare supporting the aged.

A significant contribution to the understanding of the aging phenotype is explained by the imbalance of inflammatory to anti-inflammatory networks, which results in a low-grade chronic pro-inflammatory state (Franceschi et al., 2000; Licastro et al., 2005). Within this perspective, healthy aging and longevity are the result of not only a lower propensity to mount an effective inflammatory response, but also an inefficient anti-inflammatory network. Elucidating these mechanisms are essential to our understanding of aging.

Interleukin (IL)-6 is a key molecule that has been implicated in pro- and anti-inflammatory action whose production increases in the body with age. IL-6 has been shown to be responsible for age-related alterations in peripheral and central nervous system (CNS) function, but the mechanism of pro- and anti-inflammatory actions have not been thoroughly examined. Studies have shown that high IL-6 levels in transgenic mice correspond to neurodegeneration and gliosis (Irizarry et al., 1997). Furthermore, IL-6 expression in the CNS has been shown to induce anorexia, inhibit long-term potentiation in hippocampal neurons, impair learning and memory, and induce hyperalgesisa (Bellinger et al., 1995; Dina et al., 2008; Papanicolaou et al., 1998). Therefore, it is hypothesized here that the neuro-pathological phenotype observed in the aging brain is influenced by the pro-inflammatory arm of IL-6. In this dissertation research, several behavioral, molecular, and genetic approaches were used to define

the signaling pathway involved in overexpression of pro-inflammatory mediated IL-6 in the brain of aged mice. The proceeding sections summarize peer-review literature concerning the properties, functions, and expression of IL-6, brain aging, and the role of IL-6 signaling in aging and neurodegeneration.

1.1 Literature cited

Bellinger, F.P., Madamba, S.G., Campbell, I.L., Siggins, G.R., 1995. Reduced long-term potentiation in the dentate gyrus of transgenic mice with cerebral overexpression of interleukin-6. Neuroscience Letters 198, 95-98.

Candore, G., Colonna-Romano, G., Lio, D., Caruso, C., 2003. Immunological and Immunogenetic markers of successful and unsuccessful ageing. Advances in Cell Aging and Gerontology 13, 29-45.

Dina, O.A., Green, P.G., Levine, J.D., 2008. Role of interleukin-6 in chronic muscle hyperalgesic priming. Neuroscience 152, 521-525.

Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., De Benedictis, G., 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci 908, 208-218.

Irizarry, M.C., Soriano, F., McNamara, M., Page, K.J., Schenk, D., Games, D., Hyman, B.T., 1997. Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. The Journal of Neuroscience 17, 7053-7059.

Licastro, F., Candore, G., Lio, D., Porcellini, E., Colonna-Romano, G., Franceschi, C., Caruso, C., 2005. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immunity & Ageing 2, 8.

Mariani, L., Turchetti, G., Franceschi, C., 2003. Chronic antigenic stress, immunosenescence and human survivorship over the 3 last centuries: heuristic value of a mathematical model. Mechanisms of Ageing and Development 124, 453-458.

Papanicolaou, D.A., Wilder, R.L., Manolagas, S.C., Chrousos, G.P., 1998. The pathophysiologic roles of interleukin-6 in human disease. Ann Intern Med 128, 127-137.

Vasto, S., Caruso, C., 2005. Immunity & Ageing: a new journal looking at ageing from an immunological point of view. Immunity and Ageing 2, 1.

Chapter 2

Literature review

2.1 History and general properties of interleukin-6

Interleukin (IL)-6 is a pleiotropic cytokine that is implicated in a multitude of systems. Tadamitsu Kishimoto, a Japanese born immunologist has been credited with the critical research that was instrumental of our current understanding of the function and structure of IL-6 (Kishimoto, 2005). Various groups throughout the world from Kishimoto, Jacques Van Snick and Jack Gauldie, were studying what was presumed unrelated growth factors at the time, identified the myriad of actions of IL-6. These factors had many names and functions, interferon β2 (Weissenbach et al., 1980; Zilberstein et al., 1986), B-cell stimulatory factor (Hirano et al., 1985), hybridoma growth factor (Brakenhoff et al., 1987), hepatocyte-stimulatory factor (Gauldie et al., 1987), and cytotoxic T-cell differentiation factor (Takai et al., 1988), to name a few. It was not until 1986 that human IL-6 was molecularly cloned, and a few years after that for the protein to be purified and utilized in studies to show that these seemingly different factors were the same molecule.

Interleukin-6 is a member of the long-chain class of four-α-helix bundle cytokine family. Human IL-6 is a single chain glycoprotein with a molecular weight ranging from 21-30 kDa, while murine IL-6 is a 22-29 kDa protein (Nakajima et al., 1985; Simpson et al., 1988). The observed heterogeneity is due to extensive post-translational modification attributable to variations in glycosylation, phosphorylation at multiple serine residues, and sulfation, depending on the cellular source (May et al., 1991; Nakajima et al., 1985). The moderate differences of post-translational modification of IL-6 have little

to no effect on its biological activity, as evident by the identical nature of recombinant and natural IL-6.

2.2 Immune-to-brain axis

Until recently, the brain was believed to be an immunologically privileged site (Neuwelt and Clark, 1978), but there is now an appreciation that the peripheral immune system communicates with the brain. The group of Nance and Greenberg at the University of Manitoba were the first to demonstrate that peripheral immune stimulation directly activated nuclei in the brain (Wan et al., 1993). It is now a widely accepted notion that there is a bi-directional communication with the immune system and the brain. The brain responds to peripheral immune stimulus through multiple immune-to-brain communication pathways to mount an appropriate immunological, physiological, and behavioral response that is an adaptive process (Dantzer et al., 1998a). Upon stimulation via an infectious pathogen, stress response, or injury, the peripheral innate immune system produces pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α via activated peripheral macrophages/monocytes (Gordon and Taylor, 2005), which act on the brain through neural, humoral and diffusive routes (Dantzer et al., 2008).

The neural pathway appears to be the main sensory pathway from abdominal organs to the brain and is primarily facilitated through vagal afferents, with input from other nerves in various sites where immunological processes occur (Goehler et al., 2000). The projections from the vagus nerve enter the brain stem at the nucleus of the solitary tract (NTS), ascend to the parabrachial nucleus, and project to nuclei in the hypothalamus and other parts of the brain (Maier et al., 1998). Earlier studies showed

“proof-of-concept” that peripheral cytokines that signaled to the brain through vagal afferents were responsible for sickness behaviors by cutting the vagal nerve prior to stimulation by LPS or cytokines (Bluthe et al., 1996; Maier et al., 1998). Humoral routes revolve around the blood-brain-barrier (BBB), a structure consisting of capillary endothelial cells and astrocytes within the brain that physically separates circulating blood from brain parenchyma and cerebral spinal fluid (CSF) to prevent the diffusion of harmful molecules (Banks, 2006). Endothelial cells of the BBB have polarized sides that respond to circulating cytokines on their luminal side and secrete immune molecules of their own via their abluminal side directly into the brain (Verma et al., 2006). Cytokines are also actively transported across the BBB through a series of energy-dependent pumps and carrier-mediated system for cytokines (Banks, 2006). The diffusive route includes circumventricular organs (CVOs), sites of the brain that are devoid of an intact BBB. Here cytokines enter directly into the brain parenchyma via the blood by volume diffusion (Quan, 2008). Once the signal reaches the brain, these cytokines activate microglia cells, which propagate the signal throughout the CNS (Rivest, 2003).

2.3 Brain immune activation

The CNS is privy to its own immune system consisting of glial cells primarily microglia, the macrophage cells of the brain, that serve a dual role in neuromodulation and engagement in immunological processes (Kettenmann et al., 2011). The extent to which cytokines are involved in CNS homeostatic or disease processes is a delicate balance that relies on the remarkable ability for microglia to adapt to the input from the periphery. While activation of the peripheral and central innate immune system produces cytokines to control infection and repair injury, abnormal production can result

in a more severe or chronic neuroinflammatory state that has the potential to shift the microglia response from physiological to pathological (Kettenmann et al., 2011).

Normally, microglia exist in a quiescent state in the healthy adult brain where they survey the microenvironment for changes and are characterized morphologically by a small soma and ramified processes for surveillance (Hanisch and Kettenmann, 2007). Small changes in the CNS microenvironment (i.e. brain injury, infectious pathogen, and pH) trigger a rapid and transient activation of microglial cells with concomitant changes in morphology, metabolism, and phenotype. A key sign of microglia activation are the ramified processes seen at resting are shortened and the cell soma swells to an “amoeboid” state (Ransohoff and Perry, 2009). This change in morphology is accompanied by a heightened metabolism which corresponds to upregulated cell surface molecules, including major histocompatibility class (MHC)-II marker, receptors for cytokines and chemokines, and several other cellular markers; as well as cytokine production, drastically changing the phenotype of the microglial cells (Perry, 1998).

The pro-inflammatory cytokines released from activated microglia act on neurons to elicit a set of adaptive responses that affect physical (conductance) and behavioral outputs, which is intended to facilitate recovery from sickness (Kelley et al., 2003). This response is designed to be expedited and reversible, however evidence suggests that dysregulation of microglial cell activity may be responsible for a discordant central response to peripheral immune signals. Understanding individual cytokine involvement in microglia is a prime focus for therapeutic intervention across various neurodegenerative diseases and CNS insults.

2.4 Behavioral and cognitive changes

Various growth factors and cytokines are produced by activated microglia in response to peripheral or local stimulation. Pro-inflammatory cytokines target neurons and elicit a number of behavioral responses that are collective called “sickness behavior”. These behaviors include lethargy, reduced food intake, decreased social interaction, and general malaise (Hart, 1988). Sickness behavior is adaptive because it aids an animal in its recovery processes such as conservation of metabolic demand for clearance of pathogens from a disease or injury (Dantzer et al., 1998b). An example of this is the motivation of feeding in a sick animal is drastically reduced to increase restfulness, when compared to a healthy animal. This re-organization of behavioral priorities observed in animals with an acute infection exemplifies a necessity to alter behavior to benefit the animal under unfortunate circumstances. Pharmacological strategies where anti-inflammatory agents (i.e. IL-10, IL-1RA) were administered via peripheral or central routes to showed that individual cytokines are in fact involved in the onset or persistence of sickness behaviors (Bluthe et al., 1999; Kelley et al., 2003; Kent et al., 1992).

In addition to sickness behaviors, other neuronal output, such as cognitive dysfunction are associated with microglia activation and cytokine production (Graeber and Streit, 2010). Neuroanatomy must be taken into account, when considering cytokines’ effect on neurophysiology. Consistent with the high density of microglia, cytokine concentrations are at their highest in the hippocampus, an area of the brain associated with learning and memory (Long et al., 1998; Schobitz et al., 1993). We understand that the hippocampus is involved in learning and memory from lesion

studies that show a severe impairment in cognitive processes (Chen et al., 1996), which agrees with electrophysiological data (D'Hooge and De Deyn, 2001). Further evidence that cytokines are crucial in altering neurophysiology is that elevated levels of IL-1β impedes performance of hippocampal-dependent learning and memory tasks such as contextual fear-conditioning and Morris water maze (Avital et al., 2003; Maier and Watkins, 1995).

2.5 IL-6 in the CNS

The function of IL-6 within the CNS is complex; IL-6 exerts both neurotrophic and neuroprotective effects, as well as mediating inflammatory processes, gliosis, and demyelination within the cellular populations (Akira et al., 1993; Scheller and Rose-John, 2006). Studies have shown transcripts of IL-6 and IL-6 receptor (IL-6R) expressed in various regions of the brain, including the hippocampus, striatum, cortex, cerebellum, hypothalamus, and brain stem (Gadient and Otten, 1995; Schobitz et al., 1993; Schobitz et al., 1992). Not only are IL-6 and IL-6R mRNAs expressed in different brain regions, they are co-localized on several neuronal types, indicating the breadth IL-6's actions throughout the CNS (Gadient and Otten, 1993; Schobitz et al., 1993).

IL-6's neurotrophic effects have been demonstrated in both *in vitro* and *in vivo* experiments. *In vitro* experiments have used the PC-12 neuronal cell line to show that IL-6 induces neurite outgrowth similar to nerve growth factor (NGF) (Marz et al., 1996), by blocking DNA fragmentation and preventing apoptosis (Kunioku et al., 2001; Umegaki et al., 1996). Similar results have been observed in primary neurons (Sarder et al., 1996). Additionally, IL-6 has been shown to protect various neuronal sub-

populations from NMDA receptor activation induced-neurotoxicity (Liu et al., 2011). IL-6 has also been implicated in neuroprotection against axotomy, ischemia, and nerve injury (Ikeda et al., 1996). However, with most things, spatial-temporal expression along with the amount of protein is important to the action of IL-6. Overproduction of IL-6 has been implicated in the enhanced intra-cellular calcium response to NMDA receptor-activation neurotoxicity in cultured granule neurons (Nelson et al., 2002). IL-6 in the hippocampus has been shown to impair glutamate receptor expression, leading to impairments in synaptic plasticity and deficits in long-term potentiation (LTP) (Gruol and Nelson, 2005)

While we know neurons respond to IL-6, major cellular contributors of IL-6 in the CNS are glial cells: astrocytes, microglia, and oligodendrocytes (Kishimoto, 2005; Taga and Kishimoto, 1997; Van Wagoner and Benveniste, 1999). Normally IL-6 is expressed at very low levels; however glial cell activation via overlapping stimuli purports the notion of the diverse functions of IL-6 and the tight cross communication between glial cells and neurons. These stimuli include brain injury (Gomes-Leal et al., 2004; Streit, 1996), ischemic conditions such as in a stroke (Hughes et al., 2002), pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α, and IFN-γ) (Vitkovic et al., 2000), pathogen associated molecular patterns (PAMPs), various neurotransmitter and neuropeptides (e.g. acetylcholine and norepinephrine,) (Eulenburg and Gomeza, 2010), as well as disease associated peptides, such as β-amyloid plaques and S100β (Koenigsknecht-Talboo and Landreth, 2005). Microglia primarily recognize all of the above stimuli, but evidence supports that they interact with astrocytes and astrocytes play a key role in neuroinflammation. Through loss of function studies, IL-6 is known to promote astrocyte

proliferation and astrocytes react with a delay, in contrast to microglia (Goswami et al., 1998).

2.6 The interleukin-6 receptor complex and recognition

Interleukin-6 expression is modulated by IL-1β and TNF-α through an IL-6-specific promoter region of nuclear factor kappa B (NF-κB) (NF-IL-6) (Akira et al., 1995). The role IL-6 has on its own regulation is extremely important when considering cellular sources and this has not been addressed in depth in the CNS. IL-6 exudes its biological effects on cells by binding its cognate receptor IL-6 receptor-α chain (IL-6R) and the recruitment of transmembrane signaling unit dimer glycoprotein 130 (gp130). The availability of the 3-dimensional structure of IL-6 has helped to construct a model of the IL-6 ligand interacting with the IL-6R and gp130, and give new insight into the process of molecular recognition and signaling. A 1.9A crystal structure of hIL-6 has been reported (Somers et al., 1997) and X-ray analysis reveals that 3-D structure of IL-6 is composed of a four helix bundle (Boulanger et al., 2003).

Importantly, it is the IL-6R that maintains the affinity for IL-6 and once binding occurs, a conformation shift recruits gp130 subunits; it is gp130 that has the intracellular machinery to facilitate further signal transduction. The gp130 subunit is shared as a hetero- or homodimer amongst the IL-6 receptor family that includes IL-6, IL-11, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and oncostatin M (OSM) (Kishimoto, 2005; Kishimoto et al., 1995). While expression of IL-6R is primarily on immune cells, gp130 is constitutively expressed across all cell types, which is consistent with the overlapping functionality of all the factors that use gp130 as a signaling subunit.

Identifying the site at which IL-6 binds/interacts with its receptor in addition to how the binding activates its receptor is critical to develop potential therapeutic strategies. Functional structure studies have identified three regions on the IL-6 molecule that are important for binding to the IL-6R and gp130. There is a “site 1” on IL-6 involved in binding to the IL-6R, which encompasses the C-terminal region of the ab-loop/N-terminal part of helix B (Ehlers et al., 1994). An interesting finding was that IL-6 does in fact associate with the gp130 subunit via site 1 interaction. Visualization of I^{125}-IL-6 to cellular membranes showed the association is only in the presence of IL-6R (Silvani et al., 1995). “Site II” on IL-6 is described as residues that are within helix A and C that also interact with gp130 (Paonessa et al., 1995). “Site III” is a second binding site for gp130 which comprises residues in the cd-loop/N-terminal end of helix D (Hammacher et al., 1994). A fourth binding site (“site 4”) determines the assembly of a functional hexameric 2:2:2 stoichiometry (IL-6, IL-6R, and gp130) of the IL-6 receptor complex (Boulanger et al., 2003; Ward et al., 1996)

2.7 Interleukin-6 receptor and gp130 and signaling: classical vs. trans

The IL-6R is found in two forms, a membrane bound form which has a transmembrane spanning region and a soluble form that does not have a transmembrane spanning region. Experiments that molecularly cloned the IL-6R showed that the membrane bound form of IL-6R has a very short cytoplasmic region and is not essential for IL-6 signal transduction (Yamasaki et al., 1988). The soluble IL-6R (sIL-6R) is strictly the extracellular region of the membrane bound form and is primarily generated by proteolytic shedding of membrane bound IL-6R by a class of enzymes TNF-α converting enzymes (TACE) or ADAM10/17 (Chalaris et al., 2010;

Franchimont et al., 2005; Mullberg et al., 1993); in certain cases sIL-6R is formed from an alternatively spliced mRNA species (Lust et al., 1995; Scheller and Rose-John, 2006). Membrane bound IL-6 receptor is implicated in the classical signaling response and sIL-6R is implicated in the *trans*-signaling response. Binding affinity of IL-6 to sIL-6R is very similar to membrane bound IL-6R (Muller-Newen et al., 1998; Schobitz et al., 1995) and it is the act of binding that recruits gp130, the non-ligand-binding signaling transducing molecule. In classical signaling IL-6 binds directly to membrane bound IL-6R and recruits gp130 signaling subunits; whereas *trans*-signaling involves the IL-6 binding to sIL-6R in extracellular matrix to form an IL-6/sIL-6R complex that has a very high binding affinity for ubiquitously expressed gp130 signaling subunits. This *trans*-signaling action confers IL-6 responsiveness to cells that do not typically respond to IL-6. An age-old debate has been the pro- versus anti-inflammatory actions of IL-6 in the body (Gadient and Otten, 1997; Scheller et al., 2011). It appears as if the signaling mechanism plays an important part in mediating the actions of IL-6 and *trans*-signaling is heavily implicated in pro-inflammatory processes and disease models (Barkhausen et al., 2011; Greenhill et al., 2011; Mees et al., 2009; Nowell et al., 2009; Rabe et al., 2008; Santer et al., 2010; Weidle et al., 2010). The principal idea of *trans*-signaling being a pro-inflammatory is the fact that cells which do not normally respond to IL-6, will recruit immune cells that respond to IL-6 to a site that was previously uninhabited with immune cells (Barrientos et al., 2011).

sIL-6R and soluble gp130 (sgp130) have varying effects on circulating IL-6. While sIL-6R acts as a potent agonist, sgp130 acts as a partial antagonist, or decoy receptor, by binding the IL-6/sIL-6R complex and prevents the binding of membrane-

bound gp130 and further signal transduction (Jostock et al., 2001). Under non-pathological conditions, sIL-6R is present in moderate levels in plasma at ~25-35 ng/mL and in brain extracts at 300-900 pg/mL (Van Wagoner and Benveniste, 1999). The activity of the IL-6/sIL-6R complex is tightly controlled by molar excess of naturally occurring sgp130 present in serum at levels of 100-300ng/mL to competitively inhibit signaling via membrane-bound gp130 (Narazaki et al., 1993).

The formation of the IL-6-IL-6R-gp130 complex is the initial step in IL-6 signaling. Upon hexameric complex formation, conformational changes lead to the dimerization of gp130 and intrinsic tyrosine kinases are autophosphorylated, resulting in the activation of Janus kinase-1 and 2 (Jak1 & Jak2). These tyrosine kinases phosphorylate the cytoplasmic region of gp130 creating recruitment sites for signal transducer and activation of transcription-3 (STAT3), a Src-homology-2 (SH2) domain-containing signaling molecule. Activated STAT3 forms a dimer, autophosphorylates, and translocates to the nucleus where it binds to inducing elements of the IL-6 promoter region. Thus the main consequence of classical IL-6 receptor action is to induce gene transcription and subsequent synthesis and secretion of IL-6 (Heinrich et al., 1998). Additionally, IL-6 activates the RAS/MAPK pathway through a gp130-associated SH2-containing protein phosphatase-2, as well as the phosphatidylinositol 3-kinase pathway which results in the transcription of IL-6 (Leonard et al., 1999).

2.8 Interleukin-6 gene expression

Numerous cell types are capable of IL-6 gene expression and protein synthesis, due to classic and *trans*-signaling mechanisms and the relative potency of various stimuli to induce IL-6 production is cell-type dependent. It is necessary to understand

the gene structure and transcription machinery that are involved in expression patterns. Whether in humans or rodents, the IL-6 gene contains four introns and five exons and the position of intron/exon boundaries, exon length, and location of cysteine residues within the protein coding region are conserved across species (Tanabe et al., 1988). There are major similarities of gene organization in humans and rodents and a number of putative cis-acting elements that are required for inducible and tissue-specific transcriptional regulation located in the 5' flanking region of the IL-6 gene (May et al., 1991; Sehgal et al., 1986). While STAT3 is the major transcriptional regulator of IL-6, other response elements include a region that encompasses a multiple response element that has homology to c-fos serum response element (May et al., 1989), two CCAATT/enhancer binding protein (C/EBP) sites NF-IL-6/IL-6DBP which are either mediated by STAT3 or CREB (Akira et al., 1992; Akira et al., 1990; Ramji et al., 1993), an AP-1 consensus site that corresponds with the MAPK pathway (Jang et al., 2008; Schuringa et al., 2001), and as stated above, a NF-κB site (Akira and Kishimoto, 1997).

The number of cell types that can express IL-6 translates to a wide range of stimulants that regulate the expression of IL-6. Activators span from physical injury, heat shock, other cytokines and growth factors, PAMPs, and environmental toxins (Scheller and Rose-John, 2006; Streit, 2000). In contrast, negative regulation of IL-6 transcription comes from suppressor of cytokine signaling (SOCS)-1 and 3. SOCS3 is a key physiological regulator of IL-6 signaling and was originally identified as a regulator of adaptive cytokine signaling pathways but has recently been shown to be important in regulating innate immune responses in macrophages (Croker et al., 2003; Niemand et al., 2003; Wang and Campbell, 2002). Cytokines such as IL-10, IL-4, IL-13, as well as

glucocorticoids are known to regulate IL-6 transcription (Heyen et al., 2000; Martinez et al., 2008).

2.9 Brain aging

With advances in our understanding of immune-to-brain communications, it has become apparent that the immune system has an important role in regulating the progression of brain aging and major deleterious alterations in behavioral output, including an exaggeration of sickness behaviors, depression, and declines in cognitive performance. Moreover, changes in the CNS's microenvironment during senescence, pre-disposes the aging brain to neurodegenerative diseases.

It is important to note that healthy aged individuals have upregulated basal levels of pro-inflammatory cytokines, which has been suggested to create a permissive state for the on-set of neurodegenerative disease (Liu and Hong, 2003). The MacArthur foundation study on successful aging was a 20-year longitudinal study that revealed a correlation between mild cognitive impairments and elevated serum IL-6 (Weaver et al., 2002). A similar study on patients with Alzheimer's and Down's syndrome showed that there is a clear correlation of heightened levels of IL-6, age, and neurodegenerative disease (Giunta et al., 2008; Warsch and Wright, 2010).

A long-standing debate on the cause for cognitive decline seen in aging had two sides, one argument was that the decline was due to neuronal loss through death (Dayan, 1970), and the other believed it was due to a change in cell size (Terry et al., 1987). While there is evidence to support both, new innovative experimental techniques, have improved our understanding of brain aging. A 1997 study in humans found a

steady decline in average brain weight starting after the age 60 (Mrak et al., 1997), while further studies showed a similar outcome of weight changes, with no significant variations in numbers of neurons in the brain of healthy aged individuals versus young individuals (Freeman et al., 2008). Rodent and non-human primate studies alike have provided further evidence that there is no global or hippocampal neuronal loss, and that there are stem cells that can proliferate and differentiate into neurons to replace lost neurons (Eriksson et al., 1998; Gazzaley et al., 1997; Rapp and Gallagher, 1996; Rapp et al., 1999). Although neuronal loss is not a significant factor in age-related processes, evidence from neurodevelopmental processes show that subtle changes in structure or microenvironment significantly affect neuronal populations, and changes in regions important for cognition may pre-dispose individuals to the onset of cognitive impairments (Casey et al., 2005; Dickstein et al., 2007).

Aging appears to cause a shift in the cytokine-mediated sickness response from an adaptive to a maladaptive response, leading to behavioral complications (Dantzer et al., 1999; Wynne et al., 2009). Microglia serve as sensitive sensors of events occurring in their microenvironment, providing the first line of defense in the CNS (Block et al., 2007). Activated microglia release immune mediators that coordinate the response of both innate and adaptive immunity to control infection, remove cell debris and promote repair (Kreutzberg, 1996). An exaggerated or prolonged activation state has been observed in aging, which has detrimental effects (Block et al., 2007).

The aged brain contains more reactive or hypersensitive microglia, which is denoted by markers of activation expressed on the surface such as CD68 and MHC-II. In animal models, a subset of microglia in reactive or ‘primed’ state constitute over 20%

of the population, when compared to 5% in adult animals (Henry et al., 2009). These primed microglia produce increased steady-state levels of cytokines, have begun their phenotypic shift towards the activated state, and have a higher cytokine secretion profile when further provoked (Combrinck et al., 2002; Deng et al., 2006; Sierra et al., 2007; Ye and Johnson, 1999b). For example, studies have shown that steady-state and LPS-induced IL-6 production is increased in aged microglia (Ye and Johnson, 1999a).

Astrocytes are derived from neuronal progenitor cells and have diverse functions, including providing structure and support to neurons, releasing neurotrophic factors, engaging in synaptic transmission, maintaining the integrity of the BBB, and interacting with microglia to improve the CNS microenvironment (Abbott et al., 2006; Pascual et al., 2012). Astrogliosis or activation of astrocytes is another characteristic of age-related glial changes and Glial fibrillary acidic protein (GFAP), a major cytoskeletal protein of astrocytes, has been used as a marker to document age-related increases in the number of astrocytes in rodents and humans. It was found that GFAP protein and mRNA displayed an age-related increase in many areas of the brain, including hippocampus and cortex (O'Callaghan and Miller, 1991). Additionally, the neurotrophic cytokine S100β which is a marker of activated astrocytes is increased with age in human cortex (Sheng et al., 1996). The increase of GFAP and S100β expression is also consistent with anatomical studies that showed astrocyte hypertrophy with age (Griffin et al., 1995).

Amplified or prolonged neuroinflammation leads to a maladaptive sickness response. Aged animals injected intraperitoneally with LPS exhibit protracted levels of IL-1β and IL-6 expression in the brain paralleled by prolonged deficits in food intake,

activity, and social behavior (Godbout et al., 2005). While it has been shown that peripheral factors negatively regulate neurogenesis and behavioral output in the aged (Villeda et al., 2011), the environment in the central compartment is paramount to behavioral output. ICV injection of LPS or gp120, a component of the AIDS virus cause prolonged sickness behavior in aged mice (Abraham et al., 2008; Huang et al., 2008). Infusing the receptor antagonist for IL-1β (IL-1RA) attenuated IL-1β gene expression and reversed the prolonged sickness behavior observed in aged animals (Abraham and Johnson, 2009), showing that there is a direct relationship between the central amplified cytokine response and exaggerated sickness behavior in the aged.

Indeed in brain aging, there is a reduction in neuroprotective factors and an increase in inflammatory mediators. Neuronal populations are particularly vulnerable to both and age-related impairments in neuronal output and cognitive function are associated from these consequences. For example, in rodent models stimulating the peripheral immune system via LPS or live *E.coli* infection causes an amplified hippocampal cytokine response that corresponds to impaired hippocampal-dependent memory in spatial and contextual tasks in older animals (Barrientos et al., 2006; Chen et al., 2008). Potential mechanisms by which cytokines cause cognitive complications, include neuroinflammatory pathways augment neuronal physical properties and alter plasticity (i.e. neurogenesis and long-term potentiation (Griffin et al., 2006). In another example where neuroinflammation is prolonged similar to aging, IL-10 knock-out mice displayed hippocampal-dependent cognitive deficits and increased dendritic atrophy in the CA1 region of the hippocampus after LPS injection (Richwine et al., 2008).

However other mechanisms contribute to behavioral deficits seen in aging. Important to note, peripheral cells contribute to IL-6 levels in the CNS, from perivascular microglia, endothelial cells within the BBB, as well as infiltrating T-cells and macrophages (Iwasaki et al., 1999; Kaplanski et al., 2003; Romano et al., 1997; Simard and Rivest, 2004). In aging, the BBB remains intact, however it may be more susceptible to disruption as there is evidence of altered physio-chemical properties and functions of enzymes, receptors, and particular carrier transport systems, and adhesion molecules such as ICAM and VCAM are implicated (Miguel-Hidalgo et al., 2007; Shah and Mooradian, 1997).

2.10 Aging, IL-6 and neurodegenerative diseases

Glia cells are the most influential in producing neuroprotective factors and inflammatory mediators, and expression of such factors changes with aging and the occurrence of neurodegenerative diseases. Accumulating evidence from both animal and human studies has indicated that chronic inflammatory processes contribute to the pathogenesis of neurodegenerative diseases and IL-6 is one of the cytokines that is strongly correlated to the on-set and progression of disease. Depending on the disease, a potential mechanism of IL-6 is that it is a major inducer of acute-phase proteins (APP) and the presence of these proteins (i.e. α1-anti-chymotrypsin, α2-macroglobulin, and C - reactive protein (CRP) are involved in altering normal processing of amyloid precursor protein (APP) plaques and tangles in Alzheimer Disease patients, Lewy bodies in Parkinson’s, and mitochondrial dysfunction in Amyotrophic lateral sclerosis (Graeber and Streit, 2010; Hofmann et al., 2009; Rogers et al., 1996).

In transgenic mouse models, where mice overexpress APP, there is a state of gliosis, inflammation, and increased IL-6 production (Benzing et al., 1999). This activation of glial cells ultimately changes the microenvironment and leads to deleterious effects on neurons. Other transgenic mouse models that overexpress IL-6 in either astrocytes (Campbell et al., 1993) or neurons (Fattori et al., 1995) allowed researchers to directly investigate the neuroprotective or neuropathological effects of IL-6 in the CNS. In mice where IL-6 is over-expressed in astrocytes, markers of astrocyte activation (i.e. S100β and GFAP) were upregulated, the BBB was disrupted, and other pro-inflammatory cytokines and APPs were increased (Campbell et al., 1993; Steffensen et al., 1994). These animals also had reduced LTP, structural changes in neurons within the hippocampus and cerebellum which directly corresponded to deficits in conditioned avoidance-learning (Bellinger et al., 1995; Campbell et al., 1993; Heyser et al., 1997). Interestingly, neuronal overexpressing (NSE-IL6) mice did not develop any of the neuronal manifestations, but developed active gliosis wherein astrocytes and microglia were recruited to neurons expressing IL-6 (Fattori et al., 1995). This action best describes the mechanism of the pro-inflammatory arm of IL-6 *trans*-signaling in the CNS.

Locally, IL-6's actions alone in the CNS are fairly weak; it is the presence of sIL-6R that greatly enhances IL-6-mediated responses (Van Wagoner and Benveniste, 1999). A model for multiple sclerosis that is pertinent to the pro-inflammatory IL-6 *trans*-signaling arm is experimental autoimmune encephalitis (EAE). In the EAE mouse model, IL-6 is upregulated in cerebral spinal fluid, which is directly correlated with glial activation, breakdown of the BBB, and the infiltration of activated autoimmune T-cells.

IL-6 *trans*-signaling is known to mediate T-cell expansion, infiltration, and T_{helper} cell development (Diehl and Rincon, 2002; McLoughlin et al., 2005). Blockade of IL-6 *trans*-signaling by injection of gp130-Fc fusion protein significantly delayed the onset of EAE in comparison to saline or isotype injected control animals. Histological evaluation revealed reduced numbers of infiltrated T-cells and macrophages in the spinal cord of gp130-Fc treated animals (Linker et al., 2008).

Giuliani and colleagues observed significant increases of serum sIL-6R in humans throughout life, suggesting that *trans*-signaling plays a role in the upregulation of self-perpetuated IL-6 levels (Giuliani et al., 2001). However an important question remains: What role do microglia and astrocytes have on elevated levels of IL-6 in the brain? The dysregulation of *trans*-signaling leads to the lack of the resolution of inflammation and chronic inflammatory states, which subsequently results in the priming of immune cell types, destruction of affected tissues, and behavioral deficits. To this point, no studies have examined the modes of IL-6 receptor signaling in cells of the brain. Studies that evaluate the effects of blocking IL-6 *trans*-signaling in the aged and the extent to which neuroinflammation and behavior are affected will shed light on these actions.

2.11 Literature cited

Abbott, N.J., Ronnback, L., Hansson, E., 2006. Astrocyte-endothelial interactions at the blood-brain barrier. Nature Reviews: Neuroscience 7, 41-53.

Abraham, J., Jang, S., Godbout, J.P., Chen, J., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging sensitizes mice to behavioral deficits induced by central HIV-1 gp120. Neurobiology of Aging 29, 614-621.

Abraham, J., Johnson, R.W., 2009. Central inhibition of interleukin-1 beta ameliorates sickness behavior in aged mice. Brain Behavior and Immunity 23, 396-401.

Akira, S., Isshiki, H., Nakajima, T., Kinoshita, S., Nishio, Y., Hashimoto, S., Natsuka, S., Kishimoto, T., 1992. A nuclear factor for the IL-6 gene (NF-IL6). Chemical Immunology 51, 299-322.

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., Kishimoto, T., 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. The EMBO Journal 9, 1897-1906.

Akira, S., Kishimoto, T., 1997. NF-IL6 and NF-kappa B in cytokine gene regulation. Advances in Immunology, pp. 1-46.

Akira, S., Taga, T., Kishimoto, T., 1993. Interleukin-6 in biology and medicine. Advances in Immunology 54, 1-78.

Akira, S., Yoshida, K., Tanaka, T., Taga, T., Kishimoto, T., 1995. Targeted disruption of the IL-6 related genes: gp130 and NF-IL-6. Immunological Reviews 148, 221-253.

Avital, A., Goshen, I., Kamsler, A., Segal, M., Iverfeldt, K., Richter-Levin, G., Yirmiya, R., 2003. Impaired interleukin-1 signaling is associated with deficits in hippocampal memory processes and neural plasticity. Hippocampus 13, 826-834.

Banks, W.A., 2006. The blood-brain barrier in psychoneuroimmunology. Neurologic Clinics 24, 413-419.

Barkhausen, T., Tschernig, T., Rosenstiel, P., van Griensven, M., Vonberg, R.P., Dorsch, M., Mueller-Heine, A., Chalaris, A., Scheller, J., Rose-John, S., Seegert, D., Krettek, C., Waetzig, G.H., 2011. Selective blockade of interleukin-6 trans-signaling

improves survival in a murine polymicrobial sepsis model. Critical Care Medicine 39, 1407-1413.

Barrientos, R.M., Frank, M.G., Crysdale, N.Y., Chapman, T.R., Ahrendsen, J.T., Day, H.E.W., Campeau, S., Watkins, L.R., Patterson, S.L., Maier, S.F., 2011. Little exercise, big effects: reversing aging and infection-induced memory deficits, and underlying processes. Journal of Neuroscience 31, 11578-11586.

Barrientos, R.M., Higgins, E.A., Biedenkapp, J.C., Sprunger, D.B., Wright-Hardesty, K.J., Watkins, L.R., Rudy, J.W., Maier, S.F., 2006. Peripheral infection and aging interact to impair hippocampal memory consolidation. Neurobiology of Aging 27, 723-732.

Bellinger, F.P., Madamba, S.G., Campbell, I.L., Siggins, G.R., 1995. Reduced long-term potentiation in the dentate gyrus of transgenic mice with cerebral overexpression of interleukin-6. Neuroscience Letters 198, 95-98.

Benzing, W.C., Wujek, J.R., Ward, E.K., Shaffer, D., Ashe, K.H., Younkin, S.G., Brunden, K.R., 1999. Evidence for glial-mediated inflammation in aged APP(SW) transgenic mice. Neurobiology of Aging 20, 581-589.

Block, M.L., Zecca, L., Hong, J.S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature Reviews: Neuroscience 8, 57-69.

Bluthe, R.M., Castanon, N., Pousset, F., Bristow, A., Ball, C., Lestage, J., Michaud, B., Kelley, K.W., Dantzer, R., 1999. Central injection of IL-10 antagonizes the behavioural effects of lipopolysaccharide in rats. Psychoneuroendocrinology 24, 301-311.

Bluthe, R.M., Michaud, B., Kelley, K.W., Dantzer, R., 1996. Vagotomy blocks behavioural effects of interleukin-1 injected via the intraperitoneal route but not via other systemic routes. Neuroreport 7, 2823-2827.

Boulanger, M.J., Chow, D.C., Brevnova, E.E., Garcia, K.C., 2003. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. Science 300, 2101-2104.

Brakenhoff, J.P., de Groot, E.R., Evers, R.F., Pannekoek, H., Aarden, L.A., 1987. Molecular cloning and expression of hybridoma growth factor in Escherichia coli. Journal of Immunology 139, 4116-4121.

Campbell, I.L., Abraham, C.R., Masliah, E., Kemper, P., Inglis, J.D., Oldstone, M.B.A., Mucke, L., 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin-6. Proceedings of the National Academy of Sciences of the United States of America 90, 10061-10065.

Casey, B.J., Tottenham, N., Liston, C., Durston, S., 2005. Imaging the developing brain: what have we learned about cognitive development? Trends in cognitive sciences 9, 104-110.

Chalaris, A., Gewiese, J., Paliga, K., Fleig, L., Schneede, A., Krieger, K., Rose-John, S., Scheller, J., 2010. ADAM17-mediated shedding of the IL6R induces cleavage of the membrane stub by gamma-secretase. Biochimica et biophysica acta 1803, 234-245.

Chen, C., Kim, J.J., Thompson, R.F., Tonegawa, S., 1996. Hippocampal lesions impair contextual fear conditioning in two strains of mice. Behavioral Neuroscience 110, 1177-1180.

Chen, J., Buchanan, J.B., Sparkman, N.L., Godbout, J.P., Freund, G.G., Johnson, R.W., 2008. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behavior and Immunity 22, 301-311.

Combrinck, M.I., Perry, V.H., Cunningham, C., 2002. Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease. Neuroscience 112, 7-11.

Croker, B.A., Krebs, D.L., Zhang, J.G., Wormald, S., Willson, T.A., Stanley, E.G., Robb, L., Greenhalgh, C.J., Forster, I., Clausen, B.E., Nicola, N.A., Metcalf, D., Hilton, D.J., Roberts, A.W., Alexander, W.S., 2003. SOCS3 negatively regulates IL-6 signaling in vivo. Nature Immunology 4, 540-545.

D'Hooge, R., De Deyn, P.P., 2001. Applications of the Morris water maze in the study of learning and memory. Brain Research Reviews 36, 60-90.

Dantzer, R., Aubert, A., Bluthe, R.M., Gheusi, G., Cremona, S., Laye, S., Konsman, J.P., Parnet, P., Kelley, K.W., 1999. Mechanisms of the behavioural effects of cytokines. Cytokines, Stress, and Depression 461, 83-105.

Dantzer, R., Bluthe, R.M., Gheusi, G., Cremona, S., Laye, S., Parnet, P., Kelley, K.W., 1998a. Molecular basis of sickness behavior. In: Kluger, M.J., Bartfai, T., Dinarello, C.A. (Eds.), Molecular Mechanisms of Fever, pp. 132-138.

Dantzer, R., Bluthe, R.M., Laye, S., Bret-Dibat, J.L., Parnet, P., Kelley, K.W., 1998b. Cytokines and sickness behavior. Neuroimmunomodulation 840, 586-590.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature Reviews: Neuroscience 9, 46-57.

Dayan, A.D., 1970. Quantitative histological studies on the aged human brain. I. Senile plaques and neurofibrillary tangles in "normal" patients. Acta Neuropathologica 16, 85-94.

Deng, X.H., Bertini, G., Xu, Y.Z., Yan, Z., Bentivoglio, M., 2006. Cytokine-induced activation of glial cells in the mouse brain is enhanced at an advanced age. Neuroscience 141, 645-661.

Dickstein, D.L., Kabaso, D., Rocher, A.B., Luebke, J.I., Wearne, S.L., Hof, P.R., 2007. Changes in the structural complexity of the aged brain. Aging cell 6, 275-284.

Diehl, S., Rincon, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. Molecular Immunology 39, 531-536.

Ehlers, M., Grotzinger, J., deHon, F.D., Mullberg, J., Brakenhoff, J.P., Liu, J., Wollmer, A., Rose-John, S., 1994. Identification of two novel regions of human IL-6 responsible for receptor binding and signal transduction. Journal of Immunology 153, 1744-1753.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., Gage, F.H., 1998. Neurogenesis in the adult human hippocampus. Nature Medicine 4, 1313-1317.

Eulenburg, V., Gomeza, J., 2010. Neurotransmitter transporters expressed in glial cells as regulators of synapse function. Brain Research reviews 63, 103-112.

Fattori, E., Lazzaro, D., Musiani, P., Modesti, A., Alonzi, T., Ciliberto, G., 1995. IL-6 expression in neurons of transgenic mice causes reactive astrocytosis and increase in ramified microglial cells but no neuronal damage. The European Journal of Neuroscience 7, 2441-2449.

Franchimont, N., Lambert, C., Huynen, P., Ribbens, C., Relic, B., Chariot, A., Bours, V., Piette, J., Merville, M.P., Malaise, M., 2005. Interleukin-6 receptor shedding is enhanced by interleukin-1beta and tumor necrosis factor alpha and is partially mediated by tumor necrosis factor alpha-converting enzyme in osteoblast-like cells. Arthritis and rheumatism 52, 84-93.

Freeman, S.H., Kandel, R., Cruz, L., Rozkalne, A., Newell, K., Frosch, M.P., Hedley-Whyte, E.T., Locascio, J.J., Lipsitz, L.A., Hyman, B.T., 2008. Preservation of neuronal number despite age-related cortical brain atrophy in elderly subjects without Alzheimer disease. Journal of Neuropathology and Experimental Neurology 67, 1205-1212.

Gadient, R.A., Otten, U., 1993. Differential expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat hypothalamus. Neuroscience Letters 153, 13-16.

Gadient, R.A., Otten, U., 1995. Interleukin-6 and interleukin-6 receptor mRNA expression in rat central nervous system. Annals of the New York Academy of Sciences 762, 403-406.

Gadient, R.A., Otten, U.H., 1997. Interleukin-6 (IL-6) - A molecule with both beneficial and destructive potentials. Progress in Neurobiology 52, 379-390.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., Baumann, H., 1987. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proceedings of the National Academy of Sciences of the United States of America 84, 7251-7255.

Gazzaley, A.H., Thakker, M.M., Hof, P.R., Morrison, J.H., 1997. Preserved number of entorhinal cortex layer II neurons in aged macaque monkeys. Neurobiology of Aging 18, 549-553.

Giuliani, N., Sansoni, P., Girasole, G., Vescovini, R., Passeri, G., Passeri, M., Pedrazzoni, M., 2001. Serum interleukin-6, soluble interleukin-6 receptor and soluble gp130 exhibit different patterns of age- and menopause-related changes. Experimental gerontology 36, 547-557.

Giunta, B., Fernandez, F., Nikolic, W.V., Obregon, D., Rrapo, E., Town, T., Tan, J., 2008. Inflammaging as a prodrome to Alzheimer's disease. Journal of Neuroinflammation 5, 51.

Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W., 2005. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. FASEB Journal 19, 1329.

Goehler, L.E., Gaykema, R.P.A., Hansen, M.K., Anderson, K., Maier, S.F., Watkins, L.R., 2000. Vagal immune-to-brain communication: a visceral chemosensory pathway. Autonomic Neuroscience Basic & Clinical 85, 49-59.

Gomes-Leal, W., Corkill, D.J., Freire, M.A., Picanco-Diniz, C.W., Perry, V.H., 2004. Astrocytosis, microglia activation, oligodendrocyte degeneration, and pyknosis following acute spinal cord injury. Experimental Neurology 190, 456-467.

Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. Nature Reviews Immunology 5, 953-964.

Goswami, S., Gupta, A., Sharma, S.K., 1998. Interleukin-6-mediated autocrine growth promotion in human glioblastoma multiforme cell line U87MG. Journal of Neurochemistry 71, 1837-1845.

Graeber, M.B., Streit, W.J., 2010. Microglia: biology and pathology. Acta Neuropathologica 119, 89-105.

Greenhill, C.J., Rose-John, S., Lissilaa, R., Ferlin, W., Ernst, M., Hertzog, P.J., Mansell, A., Jenkins, B.J., 2011. IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3. Journal of Immunology 186, 1199-1208.

Griffin, R., Nally, R., Nolan, Y., McCartney, Y., Linden, J., Lynch, M.A., 2006. The age-related attenuation in long-term potentiation is associated with microglial activation. Journal of Neurochemistry 99, 1263-1272.

Griffin, W.S., Yeralan, O., Sheng, J.G., Boop, F.A., Mrak, R.E., Rovnaghi, C.R., Burnett, B.A., Feoktistova, A., Van Eldik, L.J., 1995. Overexpression of the neurotrophic cytokine S100 beta in human temporal lobe epilepsy. Journal of Neurochemistry 65, 228-233.

Gruol, D.L., Nelson, T.E., 2005. Purkinje neuron physiology is altered by the inflammatory factor interleukin-6. Cerebellum 4, 198-205.

Hammacher, A., Ward, L.D., Weinstock, J., Treutlein, H., Yasukawa, K., Simpson, R.J., 1994. Structure-function analysis of human IL-6: identification of two distinct regions that are important for receptor binding. Protein science 3, 2280-2293.

Hanisch, U.K., Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature Neuroscience 10, 1387-1394.

Hart, B.L., 1988. Biological basis of the behavior of sick animals. Neuroscience and biobehavioral reviews 12, 123-137.

Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F., Graeve, L., 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochemical Journal 334, 297-314.

Henry, C.J., Huang, Y., Wynne, A.M., Godbout, J.P., 2009. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1 beta and anti-inflammatory IL-10 cytokines. Brain Behavior and Immunity 23, 309-317.

Heyen, J.R.R., Ye, S.M., Finck, B.N., Johnson, R.W., 2000. Interleukin (IL)-10 inhibits IL-6 production in microglia by preventing activation of NF-kappa B. Molecular Brain Research 77, 138-147.

Heyser, C.J., Masliah, E., Samimi, A., Campbell, I.L., Gold, L.H., 1997. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. Proceedings of the National Academy of Sciences of the United States of America 94, 1500-1505.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajima, K., Pyun, K.H., Kishimoto, T., 1985. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proceedings of the National Academy of Sciences of the United States of America 82, 5490-5494.

Hofmann, K.W., Schuh, A.F.S., Saute, J., Townsend, R., Fricke, D., Leke, R., Souza, D.O., Portela, L.V., Chaves, M.L.F., Rieder, C.R.M., 2009. Interleukin-6 serum levels in patients with Parkinson's disease. Neurochemical Research 34, 1401-1404.

Huang, Y., Henry, C.J., Dantzer, R., Johnson, R.W., Godbout, J.P., 2008. Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. Neurobiology of Aging 29, 1744-1753.

Hughes, P.M., Allegrini, P.R., Rudin, M., Perry, V.H., Mir, A.K., Wiessner, C., 2002. Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model. Journal of Cerebral Blood Flow and Metabolism 22, 308-317.

Ikeda, K., Iwasaki, Y., Shiojima, T., Kinoshita, M., 1996. Neuroprotective effect of various cytokines on developing spinal motoneurons following axotomy. Journal of the neurological sciences 135, 109-113.

Iwasaki, T., Kanda, T., Mizusawa, H., 1999. Effects of pericytes and various cytokines on integrity of endothelial monolayer originated from blood-nerve barrier: an in vitro study. Journal of Medical and Dental Sciences 46, 31-40.

Jang, S., Kelley, K.W., Johnson, R.W., 2008. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proceedings of the National Academy of Sciences of the United States of America 105, 7534-7539.

Jostock, T., Mullberg, J., Ozbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M.F., Rose-John, S., 2001. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. European Journal of Biochemistry 268, 160-167.

Kaplanski, G., Marin, V., Montero-Julian, F., Mantovani, A., Farnarier, C., 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends in Immunology 24, 25-29.

Kelley, K.W., Bluthe, R.M., Dantzer, R., Zhou, J.H., Shen, W.H., Johnson, R.W., Broussard, S.R., 2003. Cytokine-induced sickness behavior. Brain Behavior and Immunity 17, S112-S118.

Kent, S., Bluthe, R.M., Dantzer, R., Hardwick, A.J., Kelley, K.W., Rothwell, N.J., Vannice, J.L., 1992. Different receptor mechanisms mediate the pyrogenic and behavioral effects of interleukin 1. Proceedings of the National Academy of Sciences of the United States of America 89, 9117-9120.

Kettenmann, H., Hanisch, U.K., Noda, M., Verkhratsky, A., 2011. Physiology of microglia. Physiological reviews 91, 461-553.

Kishimoto, T., 2005. Interleukin-6: From basic science to medicine - 40 years in immunology. Annual Review of Immunology 23, 1-21.

Kishimoto, T., Akira, S., Narazaki, M., Taga, T., 1995. Interleukin-6 family of cytokines and gp130. Blood 86, 1243-1254.

Koenigsknecht-Talboo, J., Landreth, G.E., 2005. Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. Journal of Neuroscience 25, 8240-8249.

Kreutzberg, G.W., 1996. Microglia: A sensor for pathological events in the CNS. Trends in Neurosciences 19, 312-318.

Kunioku, H., Inoue, K., Tomida, M., 2001. Interleukin-6 protects rat PC12 cells from serum deprivation or chemotherapeutic agents through the phosphatidylinositol 3-kinase and STAT3 pathways. Neuroscience Letters 309, 13-16.

Leonard, M., Ryan, M.P., Watson, A.J., Schramek, H., Healy, E., 1999. Role of MAP kinase pathways in mediating IL-6 production in human primary mesangial and proximal tubular cells. Kidney international 56, 1366-1377.

Linker, R.A., Luhder, F., Kallen, K.J., Lee, D.H., Engelhardt, B., Rose-John, S., Gold, R., 2008. IL-6 transsignalling modulates the early effector phase of EAE and targets the blood-brain barrier. Journal of Neuroimmunology 205, 64-72.

Liu, B., Hong, J.S., 2003. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. The Journal of Pharmacology and Experimental Therapeutics 304, 1-7.

Liu, Z., Qiu, Y.H., Li, B., Ma, S.H., Peng, Y.P., 2011. Neuroprotection of interleukin-6 against NMDA-induced apoptosis and its signal-transduction mechanisms. Neurotoxicity research 19, 484-495.

Long, J.M., Kalehua, A.N., Muth, N.J., Hengemihle, J.M., Jucker, M., Calhoun, M.E., Ingram, D.K., Mouton, P.R., 1998. Stereological estimation of total microglia number in mouse hippocampus. Journal of Neuroscience Methods 84, 101-108.

Lust, J.A., Jelinek, D.F., Donovan, K.A., Frederick, L.A., Huntley, B.K., Braaten, J.K., Maihle, N.J., 1995. Sequence, expression and function of an mRNA encoding a soluble form of the human interleukin-6 receptor (sIL-6R). Current topics in microbiology and immunology 194, 199-206.

Maier, S.F., Goehler, L.E., Fleshner, M., Watkins, L.R., 1998. The role of the vagus nerve in cytokine-to-brain communication. Neuroimmunomodulation 840, 289-300.

Maier, S.F., Watkins, L.R., 1995. Intracerebroventricular interleukin-1 receptor antagonist blocks the enhancement of fear conditioning and interference with escape produced by inescapable shock. Brain Research 695, 279-282.

Martinez, F.O., Sica, A., Mantovani, A., Locati, M., 2008. Macrophage activation and polarization. Frontiers in Bioscience 13, 453-461.

Marz, P., Gadient, R.A., Otten, U., 1996. Expression of interleukin-6 receptor (IL-6R) and gp130 mRNA in PC12 cells and sympathetic neurons: Modulation by tumor necrosis factor alpha (TNF-alpha). Brain Research 706, 71-79.

May, L.T., Shaw, J.E., Khanna, A.K., Zabriskie, J.B., Sehgal, P.B., 1991. Marked cell-type-specific differences in glycosylation of human interleukin-6. Cytokine 3, 204-211.

May, L.T., Torcia, G., Cozzolino, F., Ray, A., Tatter, S.B., Santhanam, U., Sehgal, P.B., Stern, D., 1989. Interleukin-6 gene expression in human endothelial cells: RNA start sites, multiple IL-6 proteins and inhibition of proliferation. Biochemical and biophysical research communications 159, 991-998.

McLoughlin, R.M., Jenkins, B.J., Grail, D., Williams, A.S., Fielding, C.A., Parker, C.R., Ernst, M., Topley, N., Jones, S.A., 2005. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proceedings of the National Academy of Sciences of the United States of America 102, 9589-9594.

Mees, S.T., Toellner, S., Marx, K., Faendrich, F., Kallen, K.J., Schroeder, J., Haier, J., Kahlke, V., 2009. Inhibition of interleukin-6-transsignaling via gp130-Fc in hemorrhagic shock and sepsis. Journal of Surgical Research 157, 235-242.

Miguel-Hidalgo, J.J., Nithuairisg, S., Stockmeier, C., Rajkowska, G., 2007. Distribution of ICAM-1 immunoreactivity during aging in the human orbitofrontal cortex. Brain, Behavior, and Immunity 21, 100-111.

Mrak, R.E., Griffin, S.T., Graham, D.I., 1997. Aging-associated changes in human brain. Journal of neuropathology and experimental neurology 56, 1269-1275.

Mullberg, J., Schooltink, H., Stoyan, T., Gunther, M., Graeve, L., Buse, G., Mackiewicz, A., Heinrich, P.C., Rose-John, S., 1993. The soluble interleukin-6 receptor is generated by shedding. European Journal of Immunology 23, 473-480.

Muller-Newen, G., Kuster, A., Hemmann, U., Keul, R., Horsten, U., Martens, A., Graeve, L., Wijdenes, J., Heinrich, P.C., 1998. Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses. Journal of Immunology 161, 6347-6355.

Nakajima, K., Hirano, T., Takatsuki, F., Sakaguchi, N., Yoshida, N., Kishimoto, T., 1985. Physicochemical and functional properties of murine B cell-derived B cell growth factor II (WEHI-231-BCGF-II). Journal of Immunology 135, 1207-1212.

Narazaki, M., Yasukawa, K., Saito, T., Ohsugi, Y., Fukui, H., Koishihara, Y., Yancopoulos, G.D., Taga, T., Kishimoto, T., 1993. Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. Blood 82, 1120-1126.

Nelson, T.E., Ur, C.L., Gruol, D.L., 2002. Chronic interleukin-6 exposure alters electrophysiological properties and calcium signaling in developing cerebellar purkinje neurons in culture. Journal of Neurophysiology 88, 475-486.

Neuwelt, E.A., Clark, W.K., 1978. Unique aspects of central nervous system immunology. Neurosurgery 3, 419-430.

Niemand, C., Nimmesgern, A., Haan, S., Fischer, P., Schaper, F., Rossaint, R., Heinrich, P.C., Muller-Newen, G., 2003. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. Journal of Immunology 170, 3263-3272.

Nowell, M.A., Williams, A.S., Carty, S.A., Scheller, J., Hayes, A.J., Jones, G.W., Richards, P.J., Slinn, S., Ernst, M., Jenkins, B.J., Topley, N., Rose-John, S., Jones, S.A., 2009. Therapeutic Targeting of IL-6 Trans Signaling Counteracts STAT3 Control of Experimental Inflammatory Arthritis. Journal of Immunology 182, 613-622.

O'Callaghan, J.P., Miller, D.B., 1991. The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. Neurobiology of Aging 12, 171-174.

Paonessa, G., Graziani, R., De Serio, A., Savino, R., Ciapponi, L., Lahm, A., Salvati, A.L., Toniatti, C., Ciliberto, G., 1995. Two distinct and independent sites on IL-6 trigger gp 130 dimer formation and signalling. The EMBO Journal 14, 1942-1951.

Pascual, O., Ben Achour, S., Rostaing, P., Triller, A., Bessis, A., 2012. Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proceedings of the National Academy of Sciences of the United States of America 109, E197-205.

Perry, V.H., 1998. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. Journal of Neuroimmunology 90, 113-121.

Quan, N., 2008. Immune-to-brain signaling: How important are the blood-brain barrier-independent pathways? Molecular Neurobiology 37, 142-152.

Rabe, B., Chalaris, A., May, U., Waetzig, G.H., Seegert, D., Williams, A.S., Jones, S.A., Rose-John, S., Scheller, J., 2008. Transgenic blockade of interleukin 6 transsignaling abrogates inflammation. Blood 111, 1021-1028.

Ramji, D.P., Vitelli, A., Tronche, F., Cortese, R., Ciliberto, G., 1993. The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBP delta/NF-IL6 beta, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. Nucleic acids research 21, 289-294.

Ransohoff, R.M., Perry, V.H., 2009. Microglial physiology: Unique stimuli, specialized responses. Annual Review of Immunology 27, 119-145.

Rapp, P.R., Gallagher, M., 1996. Preserved neuron number in the hippocampus of aged rats with spatial learning deficits. Proceedings of the National Academy of Sciences of the United States of America 93, 9926-9930.

Rapp, P.R., Stack, E.C., Gallagher, M., 1999. Morphometric studies of the aged hippocampus: I. Volumetric analysis in behaviorally characterized rats. Journal of Comparative Neurology 403, 459-470.

Richwine, A.F., Parkin, A.O., Buchanan, J.B., Chen, J., Markham, J.A., Juraska, J.M., Johnson, R.W., 2008. Architectural changes to CA1 pyramidal neurons in adult and aged mice after peripheral immune stimulation. Psychoneuroendocrinology 33, 1369-1377.

Rivest, S., 2003. Molecular insights on the cerebral innate immune system. Brain Behavior and Immunity 17, 13-19.

Rogers, J., Webster, S., Lue, L.F., Brachova, L., Civin, W.H., Emmerling, M., Shivers, B., Walker, D., McGeer, P., 1996. Inflammation and Alzheimer's disease pathogenesis. Neurobiology of Aging 17, 681-686.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., vanHinsbergh, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., Mantovani, A., 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity 6, 315-325.

Santer, F.R., Malinowska, K., Culig, Z., Cavarretta, I.T., 2010. Interleukin-6 trans-signalling differentially regulates proliferation, migration, adhesion and maspin expression in human prostate cancer cells. Endocrine-Related Cancer 17, 241-253.

Sarder, M., Abe, K., Saito, H., Nishiyama, N., 1996. Comparative effect of IL-2 and IL-6 on morphology of cultured hippocampal neurons from fetal rat brain. Brain Research 715, 9-16.

Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 1813, 878-888.

Scheller, J., Rose-John, S., 2006. Interleukin-6 and its receptor: from bench to bedside. Medical Microbiology and Immunology 195, 173-183.

Schobitz, B., Dekloet, E.R., Sutanto, W., Holsboer, F., 1993. Cellular localization of the interleukin-6 mRNA and interleukin-6 receptor mRNA in rat brain. European Journal of Neuroscience 5, 1426-1435.

Schobitz, B., Pezeshki, G., Pohl, T., Hemmann, U., Heinrich, P.C., Holsboer, F., Reul, J., 1995. Soluble interleukin-6 (IL-6) receptor augments central effects of IL-6 in-vivo. FASEB Journal 9, 659-664.

Schobitz, B., Voorhuis, D.A.M., Dekloet, E.R., 1992. Localization of interleukin-6 messenger-rna and interleukin-6 receptor messenger-RNA in rat-brain. Neuroscience Letters 136, 189-192.

Schuringa, J.J., Timmer, H., Luttickhuizen, D., Vellenga, E., Kruijer, W., 2001. c-Jun and c-Fos cooperate with STAT3 in IL-6-induced transactivation of the IL-6 respone element (IRE). Cytokine 14, 78-87.

Sehgal, P.B., Zilberstein, A., Ruggieri, R.M., May, L.T., Ferguson-Smith, A., Slate, D.L., Revel, M., Ruddle, F.H., 1986. Human chromosome 7 carries the beta 2 interferon gene. Proceedings of the National Academy of Sciences of the United States of America 83, 5219-5222.

Shah, G.N., Mooradian, A.D., 1997. Age-related changes in the blood-brain barrier. Experimental gerontology 32, 501-519.

Sheng, J.G., Mrak, R.E., Rovnaghi, C.R., Kozlowska, E., Van Eldik, L.J., Griffin, W.S., 1996. Human brain S100 beta and S100 beta mRNA expression increases with age: pathogenic implications for Alzheimer's disease. Neurobiology of Aging 17, 359-363.

Sierra, A., Gottfried-Blackmore, A.C., McEwen, B.S., Bulloch, K., 2007. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia 55, 412-424.

Silvani, A., Ferrari, G., Paonessa, G., Toniatti, C., Parmiani, G., Colombo, M.P., 1995. Down-regulation of interleukin 6 receptor alpha chain in interleukin 6 transduced melanoma cells causes selective resistance to interleukin 6 but not to oncostatin M. Cancer research 55, 2200-2205.

Simard, A.R., Rivest, S., 2004. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. FASEB Journal 18, 998.

Simpson, R.J., Moritz, R.L., Rubira, M.R., Van Snick, J., 1988. Murine hybridoma/plasmacytoma growth factor. Complete amino-acid sequence and relation to human interleukin-6. European Journal of Biochemistry 176, 187-197.

Somers, W., Stahl, M., Seehra, J.S., 1997. 1.9 A crystal structure of interleukin 6: implications for a novel mode of receptor dimerization and signaling. The EMBO journal 16, 989-997.

Steffensen, S.C., Campbell, I.L., Henriksen, S.J., 1994. Site-specific hippocampal pathophysiology due to cerebral overexpression of interleukin-6 in transgenic mice. Brain Research 652, 149-153.

Streit, W.J., 1996. The role of microglia in brain injury. Neurotoxicology 17, 671-678.

Streit, W.J., 2000. Microglial response to brain injury: A brief synopsis. Toxicologic Pathology 28, 28-30.

Taga, T., Kishimoto, T., 1997. gp130 and the interleukin-6 family of cytokines. Annual Review of Immunology 15, 797-819.

Takai, Y., Wong, G.G., Clark, S.C., Burakoff, S.J., Herrmann, S.H., 1988. B cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes. Journal of Immunology 140, 508-512.

Tanabe, O., Akira, S., Kamiya, T., Wong, G.G., Hirano, T., Kishimoto, T., 1988. Genomic structure of the murine IL-6 gene. High degree conservation of potential regulatory sequences between mouse and human. Journal of Immunology 141, 3875-3881.

Terry, R.D., DeTeresa, R., Hansen, L.A., 1987. Neocortical cell counts in normal human adult aging. Annals of Neurology 21, 530-539.

Umegaki, H., Yamada, K., Naito, M., Kameyama, T., Iguchi, A., Nabeshima, T., 1996. Protective effect of interleukin-6 against the death of PC12 cells caused by serum deprivation or by the addition of a calcium ionophore. Biochemical Pharmacology 52, 911-916.

Van Wagoner, N.J., Benveniste, E.N., 1999. Interleukin-6 expression and regulation in astrocytes. Journal of Neuroimmunology 100, 124-139.

Verma, S., Nakaoke, R., Dohgu, S., Banks, W.A., 2006. Release of cytokines by brain endothelial cells: A polarized response to lipopolysaccharide. Brain, Behavior, and Immunity 20, 449-455.

Villeda, S.A., Luo, J., Mosher, K.I., Zou, B., Britschgi, M., Bieri, G., Stan, T.M., Fainberg, N., Ding, Z., Eggel, A., Lucin, K.M., Czirr, E., Park, J.S., Couillard-Despres, S., Aigner, L., Li, G., Peskind, E.R., Kaye, J.A., Quinn, J.F., Galasko, D.R., Xie, X.S., Rando, T.A., Wyss-Coray, T., 2011. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. Nature 477, 90-94.

Vitkovic, L., Konsman, J.P., Bockaert, J., Dantzer, R., Homburger, V., Jacque, C., 2000. Cytokine signals propagate through the brain. Molecular Psychiatry 5, 604-615.

Wan, W., Janz, L., Vriend, C.Y., Sorensen, C.M., Greenberg, A.H., Nance, D.M., 1993. Differential induction of c-Fos immunoreactivity in hypothalamus and brain stem nuclei following central and peripheral administration of endotoxin. Brain research bulletin 32, 581-587.

Wang, J., Campbell, I.L., 2002. Cytokine signaling in the brain: putting a SOCS in it? Journal of Neuroscience Research 67, 423-427.

Ward, L.D., Hammacher, A., Howlett, G.J., Matthews, J.M., Fabri, L., Moritz, R.L., Nice, E.C., Weinstock, J., Simpson, R.J., 1996. Influence of interleukin-6 (IL-6) dimerization on formation of the high affinity hexameric IL-6.receptor complex. The Journal of Biological Chemistry 271, 20138-20144.

Warsch, J.R., Wright, C.B., 2010. The aging mind: vascular health in normal cognitive aging. Journal of the American Geriatrics Society 58 Suppl 2, S319-324.

Weaver, J.D., Huang, M.H., Albert, M., Harris, T., Rowe, J.W., Seeman, T.E., 2002. Interleukin-6 and risk of cognitive decline - MacArthur studies of successful aging. Neurology 59, 371-378.

Weidle, U.H., Klostermann, S., Eggle, D., Kruger, A., 2010. Interleukin 6/interleukin 6 receptor interaction and its role as a therapeutic target for treatment of cachexia and cancer. Cancer Genomics Proteomics 7, 287-302.

Weissenbach, J., Chernajovsky, Y., Zeevi, M., Shulman, L., Soreq, H., Nir, U., Wallach, D., Perricaudet, M., Tiollais, P., Revel, M., 1980. Two interferon mRNAs in human fibroblasts: in vitro translation and Escherichia coli cloning studies. Proceedings of the National Academy of Sciences of the United States of America 77, 7152-7156.

Wynne, A.M., Henry, C.J., Godbout, J.P., 2009. Immune and behavioral consequences of microglial reactivity in the aged brain. Integrative and Comparative Biology 49, 254-266.

Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., Kishimoto, T., 1988. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. Science 241, 825-828.

Ye, S.-M., Johnson, R.W., 1999a. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Ye, S.M., Johnson, R.W., 1999b. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Zilberstein, A., Ruggieri, R., Korn, J.H., Revel, M., 1986. Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth-stimulatory cytokines. The EMBO Journal 5, 2529-2537.

Chapter 3

Interleukin-6 is involved in microglia priming and the exaggerated LPS-induced sickness response in aged mice

3.1 Abstract

Interleukin (IL)-6 is increased in the brain of healthy aged animals and during peripheral infection, excessive production of pro-inflammatory cytokines in the aged brain from primed microglia induces behavioral pathologies. The pro-inflammatory cytokine IL-6 increases in the brain with age, but if it has a role in microglia priming is not known. The purpose of this study was to examine the functional role of IL-6 on microglia priming. In an initial study, BV.2 microglia were treated with soluble IL-6R (sIL-6R) and IL-6 alone or in combination and MHC-II expression was assessed. While subsequent studies isolated microglia from adult (3-5 month) and aged (22-24 month) C57BL6/J (IL-$6^{+/+}$) and IL-6 knock-out (IL-$6^{-/-}$) mice showed significantly less MHC-II expression in aged IL-$6^{-/-}$ compared to IL-$6^{+/+}$ counterparts. Additionally, adult and aged IL-$6^{+/+}$ and IL-$6^{-/-}$ were injected with lipopolysaccharide (LPS) to simulate a peripheral infection and sickness behaviors (spontaneous locomotor behavior, body weight changes, and food intake) and hippocampal cytokine gene expression were measured over a 24 h period. Aged IL-$6^{-/-}$ animals were resilient to exaggerated LPS-induced reductions in locomotor behavior and changes in body weight. Additionally, the age-associated baseline increase of IL-1β gene expression was ablated in aged IL-$6^{-/-}$ mice, suggesting IL-6 is a key driver of cytokine activity from primed microglia in the aged brain. Taken together, the present study suggests IL-6 expression throughout life is

involved in microglia priming and the increased amounts of IL-6 following peripheral LPS challenge is involved in the exaggerated and protracted sickness behavior response in the aged.

3.2 Introduction

Peripheral immune stimulation causes the production of pro-inflammatory cytokines, such as IL-6 and the signal for these cytokines are transported to the brain via neural and humoral pathways (Maier et al., 1998; Quan, 2008). In the brain, microglial cells react to the signal from the periphery and produce pro-inflammatory cytokines. These cytokines target neurons to elicit a sickness behavior response that is adaptive (Dantzer et al., 1998). Microglia appear to be the main cell in the brain that express the IL-6 receptor and potently secrete IL-6 during peripheral immune stimulation. IL-$6^{-/-}$ animals have shown an overall decrease in the number of activated brain macrophages associated with lesions, suggesting a role for IL-6 in the orchestration of CNS inflammation (Penkowa et al., 1999). IL-6 production in the CNS is also implicated in a myriad of behavioral pathways (Banks et al., 1994; Campbell et al., 1993; Vallieres et al., 1997). Recently we have shown that IL-6 in the brain plays a pivotal role in mediating lipopolysaccharide (LPS)-induced sickness behavior (Burton et al., 2011) as well as cognitive deficits (Sparkman et al., 2006) in adult animals.

IL-6 is also upregulated in an array of neurodegenerative diseases (Gruol and Nelson, 1997; Licastro et al., 2003; Muller et al., 1998), indicating that IL-6 plays a crucial role as a mediator of processes in the central nervous system during disease and aging.

Microglia are derived from early myeloid lineage cells and represent approximately 10–12% of the total CNS cell population (Ginhoux et al., 2010). Normally, microglia are quiescent and in an immune surveillance state in the absence of any stimuli (Nimmerjahn et al., 2005). Once activated, microglia possess a macrophage-like phenotype including, inflammatory cytokine production, phagocytosis, and antigen presentation (Ransohoff and Perry, 2009). This neuroinflammatory process is normally transient with microglia returning to a resting state as the immune stimulus is resolved. However, in various brain environments from neurodegenerative disease to aging it is proposed that elements render microglia in a “primed” or “reactive” state, wherein a subsequent local or peripheral immune challenge causes an exaggerated and protracted cytokine production (Dilger and Johnson, 2008; Godbout and Johnson, 2009).

Markers of primed microglia such as major histocompatibility complex (MHC) classII (MHC-II) and CD68 are increased in the brain during normal aging (Frank et al., 2006a; Ogura et al., 1994; Wong et al., 2005). *Ex-vivo* or peripheral immune stimulation results in an exaggerated cytokine response in microglia that express higher levels of MHC-II in aged animals (Henry et al., 2009; Njie et al., 2012). Furthermore, some studies support a link between IL-6 and MHC-II expression in monocyte-derived cells (Shafer et al., 2002; Vassiliadis and Papadopoulos, 1995). Evidence from previous studies in our lab show that microglia from aged animals are potent producers of IL-6 (Godbout and Johnson, 2004; Ye and Johnson, 1999), and that decreasing the amount of IL-6 after or during peripheral stimulation decreases other pro-inflammatory cytokines as well as improves free radical production (Godbout et al., 2004). Further findings from

our and other labs show that exaggerated pro-inflammatory cytokines interact with the brain microenvironment and exhibit more severe sickness behaviors (Godbout et al., 2005), depressive-like behaviors (Godbout et al., 2008), and deficits in hippocampal-dependent learning and memory compared with younger cohorts (Barrientos et al., 2006; Chen et al., 2008). Interestingly, when the pro-inflammatory arm of IL-6 is inhibited during peripheral immune stimulation, aged animals are refractory to cognitive deficits (Burton and Johnson, 2011). Although these data lay the groundwork to support the notion that microglial priming plays a central role in exaggerated neuroinflammation and behavioral deficits, IL-6-specific involvement during aging has yet to be determined.

3.3 Materials and methods

BV.2 microglial cell culture

The murine microglia cell line, BV.2 (a gift from Linda Van Eldik, Northwestern University, Evanston, IL) and have been used as a model to investigate the neuroimmune system (Jang et al., 2008; Zorina et al., 2010). Cells were maintained in 150-cm^2 tissue culture flasks (BD Falcon, Franklin Lakes, NJ) in DMEM (Bio-Whittaker, Cambrex, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 200 mM glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator under 5% CO_2. Confluent cultures were passed by trypsinization. Cells were centrifuged (5 minutes (min) at 27°C, 200 × *g*), and culture medium was removed. In all experiments, cells were re-suspended in DMEM supplemented with 10% FBS and seeded in six-well plates (BD Falcon, Franklin Lakes,NJ) at a population of 5 × 10^5 cells per well overnight at 37°C in a humidified incubator under 5% CO_2 before

treatments. Cells were treated with sterile saline containing 0.1% BSA (vehicle) or sIL-6R (R&D systems, Minneapolis, MN) for 1 hour (h) followed by treatment with recombinant IL-6 (R&D systems, Minneapolis, MN) for 24 h. Cells were then washed with ice cold PBS and prepared for flow cytometric analysis. BV.2 microglial cells were assayed for surface markers as described previously, with a few modifications (Burton et al., 2011). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA) in a PBS-1% BSA/sodium azide solution, and incubated with anti-CD11b APC and anti- MHC-II PE (eBioscience, San Diego, CA), fluorescently labeled isotype antibodies for APC and PE (eBioscience, San Diego, CA), and unstained samples were used for controls. Expression of surface receptors was determined using a Becton-Dickinson LSR II Flow Cytometer (Red Oaks, CA). Thirty thousand events were collected and flow data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Animal Studies

Adult (3–5 months) and aged (22-24 months) male C57BL/6 (IL-$6^{+/+}$) and IL-6 knockout B6.129S2-Il6tm1 Kopf/J (IL-$6^{-/-}$) (Kopf et al., 1994) mice were used. All mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were 2-months old upon receipt. Mice were housed in polypropylene cages and maintained at 21°C under a reverse-phase 12-h light-dark cycle with *ad libitum* access to water and rodent chow. At the end of each study, mice were examined post mortem for gross signs of disease (e.g., tumors or splenomegaly). Data from mice determined to be unhealthy were excluded from the analysis (<5%). All procedures were in accordance with the National

Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Experimental protocols

Mice were handled 1-2 min each day for 7 days before experimentation to acclimate them to handling. In some cases microglia from whole brain was isolated from animals. To assess the effects of LPS on sickness behavior and pro-inflammatory gene expression in the hippocampus, mice were injected i.p. with sterile saline or 3.30 mg/kg BW (100 µg) LPS (serotype 0127:B8, obtained from Sigma, St. Louis, MO) and killed by CO_2 asphyxiation 24 h later. Blood samples were collected via cardiac puncture into EDTA-coated syringes to obtain plasma, and the brain was rapidly removed and dissected to obtain hippocampal tissue. Hippocampal tissue was snap frozen in liquid nitrogen and stored at -80^{o} C for later analysis. In some cases, mice were evaluated for sickness behavior before killed to obtain hippocampal tissue as described above.

Microglia isolation

In experiments for flow cytometry, microglia from whole brain were isolated as described previously, with few modifications (Cardona et al., 2006; Henry et al., 2009). Mice were euthanized by CO_2 asphyxiation and whole brains were collected and stored in sterile PBS. Brains were homogenized by passage through a 70 µm cell strainer in Dulbecco's Phosphate Buffered Salt Solution (DPBS) supplemented with 0.2% glucose. Resulting homogenates were centrifuged at 600 × g for 6 min at 10^{o}C. Supernatants were removed and cell pellets were re-suspended in a 70% isotonic Percoll (GE-

healthcare, Uppsala, Sweden) supplemented with phenol red (0.01%) at room temperature. A discontinuous Percoll density gradient of 70%, 50%, 35%, and 0% isotonic Percoll was set up. The gradient was centrifuged for 20 min at 2000 × g and microglia were collected from the interphase between the 70% and 50% Percoll layers (Frank et al., 2006b). Cells were washed with DPBS and then re-suspended in PBS-0.5% BSA/ 0.01% sodium azide solution (flow buffer). The number of viable cells was determined using a hemacytometer and 0.1% trypan blue staining; each isolation yielded approximately 3 $\times 10^5$ viable cells.

Flow Cytometry

Flow cytometric analysis of microglial surface markers was performed as described previously, with a few modifications (Henry et al., 2008). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA) in flow buffer. The cells were then incubated with anti-CD11b-Allophycocyanin (APC), anti-CD45-Fluorescein isothiocyanate (FITC), and anti-MHC-II-Phychoerythrin (PE) antibodies (eBioscience, CA); fluorescently labeled isotype antibodies for APC, FITC, and PE (eBioscience, San Diego, CA), and unstained samples were used as controls. Expression of surface receptors was determined using a Becton-Dickinson LSR II Flow Cytometer (Red Oaks, CA). Thirty thousand events were collected and microglia were identified by CD11b+ and $CD45^{+low}$ expression (Ford et al., 1995). Flow data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Behavioral tests

Locomotor activity: Mice were maintained in their home cage and locomotor activity was video recorded during 5 min intervals using a camera mounted approximately 91.0 cm directly above the center of the cage floor. Tests were conducted during the dark phase (between 07:00 and 19:00) of the light/dark cycle under infrared lighting to aid video recording. Baseline behavior was taken just before LPS treatment (0 µg or 100 µg i.p.) and 4, 6, 8, and 24 h afterwards. Videos were tracked to by Ethovision (Noldus, Leesburg, VA) software, to record total distance moved. Body weight and food intake were measured at each time point over the 24 h period.

Cytokine mRNA measurement by quantitative real-time PCR

Total RNA from hippocampus was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO.) A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's protocol. Quantitative real time PCR was performed using the Applied Biosystems (Foster, CA) Assay-on Demand Gene Expression protocol as previously described (Krzyszton et al., 2008). In brief, cDNA was amplified by PCR where a target cDNA (IL-6, Mm00446190_m1; IL-1β, Mm00434228_m1; TNF-α, Mm00443258_m1; and CD68, Mm0089636_g1; MHC-II, Mm00439221_m1) and a reference cDNA (glucose-3 phosphate dehydrogenase, Mm99999915_g1) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR reactions were performed in triplicate

under the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, and results are expressed as fold difference.

Statistical analysis

All data were analyzed using the ANOVA routine in Statview and MIXED procedure of the Statistical Analysis System software (SAS Inst., Cary, NC). Data were subjected to a univariate analysis to ensure normality. BV.2 average data was subjected to a two-way ANOVA (pre-treatment × treatment). Locomotor data were and subjected to an ANOVA (age × genotype × treatment × time) using repeated measures in which time (0, 4, 6, 8, and 24 h) was a within subjects measure, and age and treatment (LPS or sterile saline) and genotype were between subjects measures, but data split into genotype graphs for ease of reading. Weight loss, food intake, microglia, and cytokine mRNA data were analyzed using a three-way ANOVA (age × genotype × treatment). Post hoc Student's *t* test of least square means was used to determine if treatment means were significantly different from one another ($p<0.05$). All data are presented as mean ± standard error of the mean (SEM).

3.4 Results

IL-6- *trans*-signaling induced MHC-II expression in BV.2 microglia

To assess if IL-6 signaling is involved in MHC-II induction on microglial cells, MHC-II cell surface expression was examined after cells were pre-treated 1 h with vehicle or sIL-6R then treated 24 h with 0, 100, or 1000 pg/mL IL-6. Figures 3.1, 3.2, and 3.3 show two-color dot blots, while Figure 3.4 is a bar graph that summarizes the data of average expression of MHC-II. Previous studies have shown IL-6 alone has an effect on MHC-II expression (Shafer et al., 2002), but recent studies implicate IL-6 *trans*-signaling in pro-inflammatory processes (Greenhill et al., 2011), and we believe this to be important in microglia activation. Figures 3.1A and B display that vehicle and sIL-6R treated microglial BV.2 cells express a similar amount of MHC-II, roughly 50% of the cells. Figure 3.2 demonstrates that treatment with 100 pg/mL IL-6 alone does not significantly induce MHC-II expression (Figure 3.2A). However pre-treatment with sIL-6R for 1 h significantly increased the induction of MHC-II expressing cells to 100 pg/mL IL-6 ($p<0.01$) (Figure 3.2B), this data displays that the IL-6 *trans*-signaling pathway is in fact implicated in activating microglia cells. Figure 3.3 indicates a ceiling effect, whereby 1000 pg/mL of IL-6 in the absence or presence of sIL-6R maximally induces MHC-II expression on microglia cells at 80-90% of the cells. These findings suggest that classic and *trans*-signaling on microglia readily regulate microglia activation or priming.

Microglia from aged IL-6$^{-/-}$ animals are refractory to priming

In aging, there is a population of microglia that are in a primed state, and MHC-II is a marker of this. Studies have found that mRNA and protein for MHC-II is basally

upregulated during aging (Frank et al., 2006a; Henry et al., 2009), and may be a major pre-disposing factor for the exaggerated pro-inflammatory cytokine production seen in aging (Henry et al., 2009). Therefore, we investigated the effects of IL-6 throughout life by isolating microglia from adult and aged IL-$6^{+/+}$ and IL-$6^{-/-}$ mice, stained for CD11b, CD45, and MHC-II, and analyzed them by flow cytometry (Figures 3.5, 3.6, and 3.7). To verify our microglia population, we profiled the isolated cells for a $CD11b^{+}/CD45^{low}$ cell population in representative two-color dot blots for adult (Figure 3.5A) and aged (Figure 3.6A) animals (Ford et al., 1995). Upon identifying microglia cells, we then gated our population for $CD11b^{+}/MHC\text{-}II^{+}$ for each age and genotype (Figures 3.5B and C and 3.6B and C). Despite the strain difference from previous studies (BALB/c vs. C57 B/6) (Henry et al., 2009) the results are consistent in that, the number of MHC-II expressing microglia is increased in IL-$6^{+/+}$ aged individuals to about 20% versus 4% in adult animals (Figure 3.7). Interestingly, the microglia from aged IL-$6^{-/-}$ animals displayed a significantly reduced number of MHC-II expressing microglia, down to roughly 11% ($p<0.05$) (Figure 3.6C). While there was no difference in MHC-II expression in IL-$6^{-/-}$ versus IL-$6^{+/+}$ adult animals (Figures 3.5B and C).

IL-6 is involved in recovery from LPS-induced sickness behavior in aged animals

Primed microglia produce exaggerated amounts of pro-inflammatory cytokines, including IL-6 that induces exacerbated and prolonged sickness behaviors. Given the results from our and others' studies (Burton et al., 2011; Nguyen et al., 2011); we investigated the effects of IL-6 and LPS-induced sickness behaviors in the aged. Adult and aged IL-$6^{+/+}$ and IL-$6^{-/-}$ animals were administered LPS i.p. and locomotor activity (Figures 3.8 and 3.9), body weight, and food intake (Figures 3.10 and 3.11) were used

as measures of sickness. As expected, LPS-treated IL-$6^{+/+}$ adult and aged animals showed a time-dependent decrease in locomotor activity ($p<0.001$). Adult and aged IL-$6^{-/-}$ animals also displayed a decrease in locomotor activity for the 4 and 6 time points (Figures 3.8 and 3.9). In Figure 3.8, LPS induced modest sickness in adult IL-$6^{+/+}$ mice where LPS-induced sickness behavior returned to baseline by 24 h post-injection, however, aged IL-$6^{+/+}$ mice were still impaired at 24 h, consistent with prior studies (Godbout et al., 2005). The LPS-induced depression of locomotor activity in IL-$6^{+/+}$ adult and aged mice was ameliorated in the IL-$6^{-/-}$ animals at the 8 h time point, an moderate difference in the adult, and a dramatic improvement in the aged (Figure 3.9). LPS-treated IL-$6^{-/-}$ aged animals were statistically lower from saline-treated animals at the 4 and 6 h time point, however the depression was not as severe as IL-$6^{+/+}$ counterparts. The initial depression of locomotor activity can be attributed to LPS-induced IL-1β production, which is implicated in the onset of sickness (Abraham and Johnson, 2009). The LPS-induced sickness response was not inhibited in adult IL-$6^{-/-}$ animals as well, however these animals exhibited a similar recovery to LPS treatment at 8 h post LPS similar to the aged animals. Additionally, IL-6 had significant mitigating effects on LPS-induced weight loss ($p<0.05$, Figure 3.10B) in aged animals, where the weight loss was not exaggerated as in the IL-$6^{+/+}$ animals. However the lack of IL-6 did not have similar effects in adult animals. Moreover, LPS-induced reductions in food intake for both IL-$6^{+/+}$ and IL-$6^{-/-}$ aged animals were significantly lower than LPS-treated adult counterparts (Figures 3.11A and 3.11B). Similar to the weight loss data the food intake data showed no difference in adult IL-$6^{-/-}$ animals. These data suggest that IL-6 is important for

maintaining sickness behavior and aged animals appear to be more sensitive to the effects of LPS-induced IL-6 production.

IL-6 is implicated in reduced levels of baseline IL-1β and MHC-II gene expression in aged animals

Previous studies have shown that MHC-II activated microglia are mainly responsible for the exaggerated production of IL-1β (Henry et al., 2009), and our earlier results suggest that a life devoid of IL-6 reduces the amount MHC-II expressed on microglia. We then proposed to assay for gene expression of markers of inflammation IL-1β, TNF-α, MHC-II, CD68, and IL-6 in hippocampal tissue 24 h after LPS treatment. We chose to use the hippocampus, because it is enriched in microglia and a region of the brain that is sensitive to LPS-induced pro-inflammatory cytokines. Our data is consistent with previous studies (Godbout et al., 2005) and shows a basal age-related upregulation of IL-1β, IL-6, and MHC-II expression seen in IL-$6^{+/+}$ animals, however in IL-$6^{-/-}$ animals, this upregulation is significantly decreased in IL-1β (Figure 3.12A) and MHC-II (Figure 3.12C). Upon LPS treatment there was no significant overall age × genotype × LPS interaction in IL-1β, TNF-α, MHC-II, or CD68 gene expression. This signifies that, while IL-6 is not the only cytokine important for microglia priming and exaggerated cytokine production, it is an integral component.

3.5 Discussion

Peripheral immune-to-brain communication causes the upregulation of various pro-inflammatory cytokines, including IL-6. During normal aging, primed microglia are responsible for the exaggerated and prolonged cytokine production and the ensuing

behavioral response during a peripheral infection (Godbout et al., 2005; Godbout and Johnson, 2009); making it a necessity to understand the involvement of individual cytokines on microglial priming and behavioral output, during aging. These data extend ours' and others' previous works, with several novel findings. First, we show that IL-6 *trans*-signaling is an important pathway for MHC-II induction (Figure 3.4). Secondly, the age-related increase of MHC-II expression was decreased in aged IL-$6^{-/-}$ microglia (Figure 3.7). Next, we showed the exaggerated sickness behavior response seen in aged animals after peripheral LPS injection was mitigated in the IL-$6^{-/-}$ animals (Figures 3.8, 3.9, 3.10B). Lastly, baseline age-related induction of IL-1β and MHC-II was decreased in aged IL-$6^{-/-}$ mice (Figures 3.12A and C). These data further demonstrate that MHC-II plays a role in microglial hyperactivity in the aged brain and indicate that IL-6 is a significant contributor to the microglia-induced exaggeration in neuroinflammation in aged mice following peripheral immune stimulation.

An important finding of this study was the age-related increase of MHC-II expression in microglia and hippocampal tissue was down-regulated in aged IL-$6^{-/-}$ animals (Figures 3.6 and 3.12C). We and other labs have previously reported an age-related increase in MHC-II gene expression in the brain of aged animals (Frank et al., 2006a; Godbout et al., 2005), but the current study provides novel evidence of an IL-6 dependent mechanism for increased MHC-II mRNA in hippocampus and protein expression on microglia isolated from aged mice. The data shows, approximately 20% of microglia from aged IL-$6^{+/+}$ mice were positive for MHC-II expression compared to 11% in the aged IL-$6^{-/-}$ animals (Figure 3.7) and the hippocampal tissue displayed half of

the baseline gene expression seen in aging (Figure 3.12C). These data indicate that IL-6 is involved in the primed phenotype in microglia from aged mice.

Microglia priming is implicated in various other models of peripheral stimulation. For instance, in the ME7 murine model of prion disease, peripheral administration of LPS induces an upregulated pro-inflammatory cytokine response in microglia, which elicits exaggerated sickness behavior, and accelerates progression of the disease (Combrinck et al., 2002; Cunningham et al., 2009). Similar studies in other rodent models of chronic neurodegenerative disease, such as amyotrophic lateral sclerosis (Nguyen et al., 2004; Nguyen et al., 2002), show the disease is responsible for a priming effect and a subsequent signal from the periphery elicits a microglial response that is greater in magnitude than individual stimuli alone. This interaction lays the basis to our understanding on how infection serves as a risk factor for exaggerated behavioral manifestations in patients with chronic neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease (Dilger and Johnson, 2008; Holmes et al., 2003).

A recent study using IL-6 knock-out animals showed that IL-6 is important for peripheral immune stimulated- sickness behavior in adult animals (Nguyen et al., 2011), while other studies that block the pro-inflammatory arm of IL-6 signaling facilitated recovery of LPS-induced sickness behavior in adult animals (Burton et al., 2011). A complementing finding of this study was that the aged IL-$6^{-/-}$ animals' sickness behavior response to peripheral LPS injection was blunted. Based on previous findings in older mice (Godbout et al., 2005), we anticipated that LPS injection would be associated with a protracted decrease in locomotor activity and an exaggeration in weight loss and a decrease in food intake over a 24 h period. As expected, there was a significant

decrease in locomotor activity in LPS treated aged IL-$6^{+/+}$ mice, (Figure 3.8), body weight (Figure 3.10A), and food intake (Figure 3.11A) 24 h after injection. Interestingly, both adult and aged IL-$6^{-/-}$ animals displayed a recovery from LPS-induced depression of locomotor activity at 8 h post LPS (Figure 3.9), a significant improvement. Body weight measurements 24 h after LPS injection showed that the aged IL-$6^{-/-}$ response was no different than the adult LPS treated groups, demonstrating that aged IL-$6^{-/-}$ animals do not show the exaggerated behavioral phenotype (Figure 3.10B). Contrary to our expectations, food intake from aged IL-$6^{-/-}$ animals was significantly decreased (Figure 3.11B); presumably because IL-1β compensates for IL-6 to act in the hypothalamus to control food intake (Harden et al., 2008). Further evidence of IL-6 as a neuromodulator have been shown in studies where adult IL-$6^{-/-}$ animals were refractory to LPS-induced deficits in the Morris water maze, while other studies from our lab have shown that blocking the pro-inflammatory arm of IL-6 in aged animals, rescued LPS-induced deficits in hippocampal-dependent contextual fear conditioning (Burton and Johnson, 2011).

A final original finding was that, baseline upregulation of gene expression of IL-1β and MHC-II observed in aging was decreased in the hippocampus of IL-$6^{-/-}$ aged animals (Figure 3.12A and C). In aging, primed microglia exhibit constitutive expression of IL-1β (Henry et al., 2009), and this is associated with neurological disease (Griffin and Mrak, 2002) and behavioral deficits (Abraham and Johnson, 2009). This downregulation displays a possible new mechanism of IL-6 - IL-1β interaction, where other studies have shown that IL-1β influences IL-6 (Cahill and Rogers, 2008), but this data provides evidence of a bi-directional interaction. Taken together, our findings

indicate that IL-6 is involved in microglial priming and the immunological and behavioral deficits seen after peripheral LPS challenge in the aged.

In conclusion, the present study demonstrates that IL-6 is involved in the pronounced age-related increase in MHC II protein in microglia. Moreover, IL-6 augments the behavioral response to peripheral LPS injection associated with exaggerated microglial activation, and induction of pro- inflammatory IL-1β in aging. These data are significant because they show the importance of IL-6 to microglial priming in the aged brain, and provides further evidence that MHC II activated microglia are significant contributors to the exaggerated neuroinflammation and behavior in aged mice following peripheral LPS challenge.

3.6 Figures

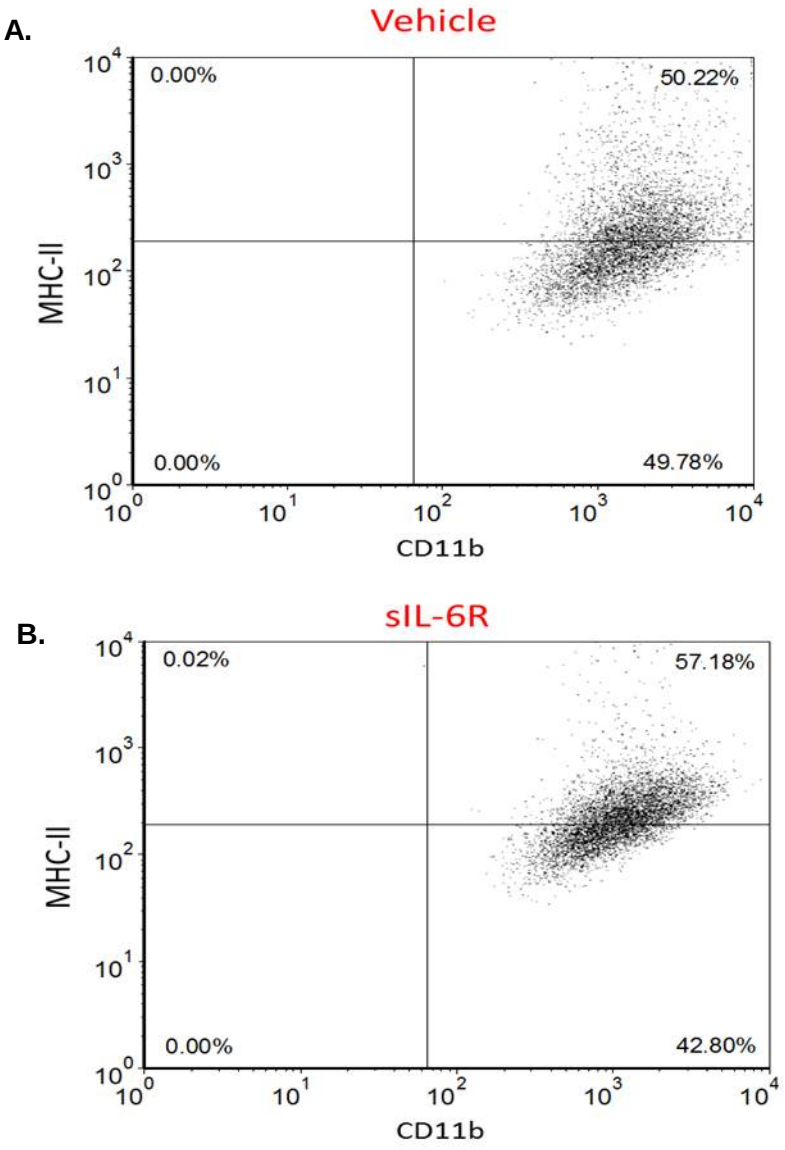


Figure 3.1: Differential expression of IL-6-induced MHC-II on microglia.
Representative two-color dot plot; cells were treated 24 h with vehicle or sIL-6R and incubated with anti-CD11b APC and anti-MHC-II PE and expression of the cell surface markers was assessed; compared with isotype and unstained controls.

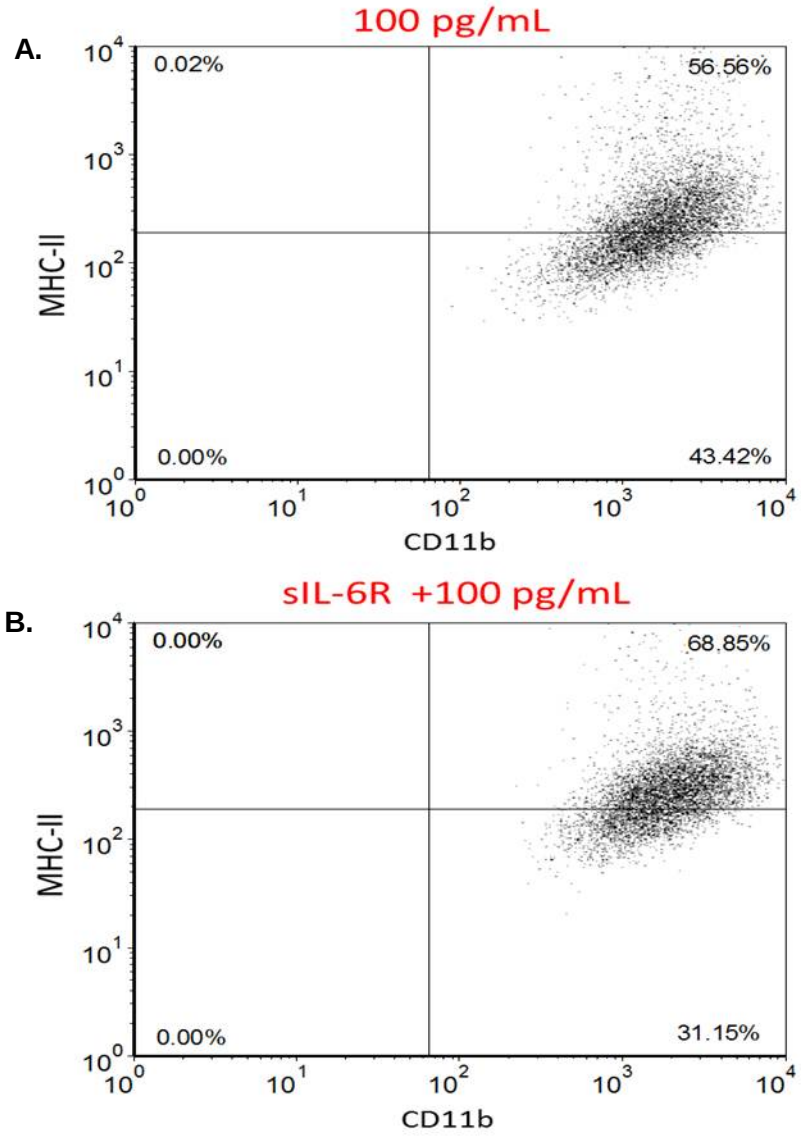


Figure 3.2: Higher expression of IL-6-induced MHC-II on microglia in the presence of sIL-6R. Representative two-color dot plot; cells were pre-treated 1 h with vehicle or sIL-6R then treated 24 h with 100 pg/mL IL-6 then and incubated with anti-CD11b APC and anti-MHC-II PE and expression of the cell surface markers were assessed; compared with isotype and unstained controls.

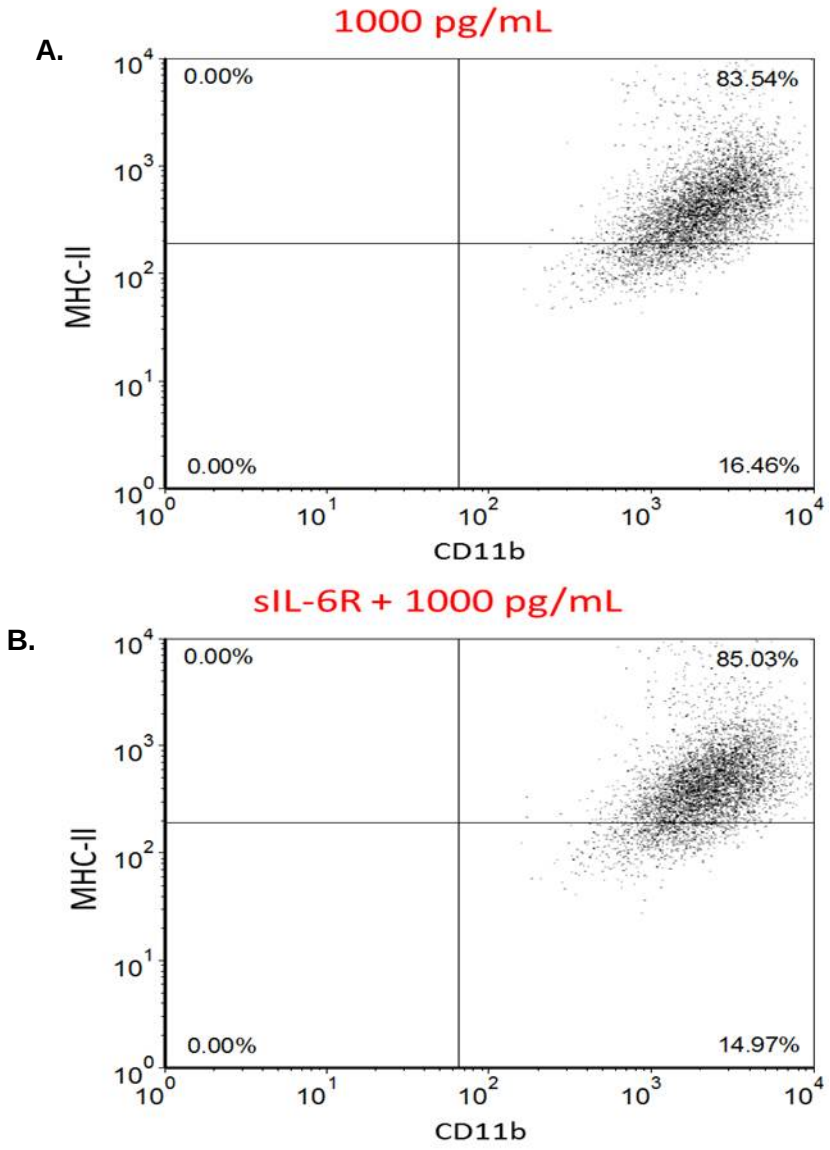


Figure 3.3: Maximal expression of IL-6-induced MHC-II on microglia. Representative two-color dot plot; cells were pre-treated 1 h with vehicle or sIL-6R then treated 24 h with 1000 pg/mL IL-6 then and incubated with anti-CD11b APC and anti-MHC-II PE and expression of the cell surface markers were assessed; compared with isotype and unstained controls.

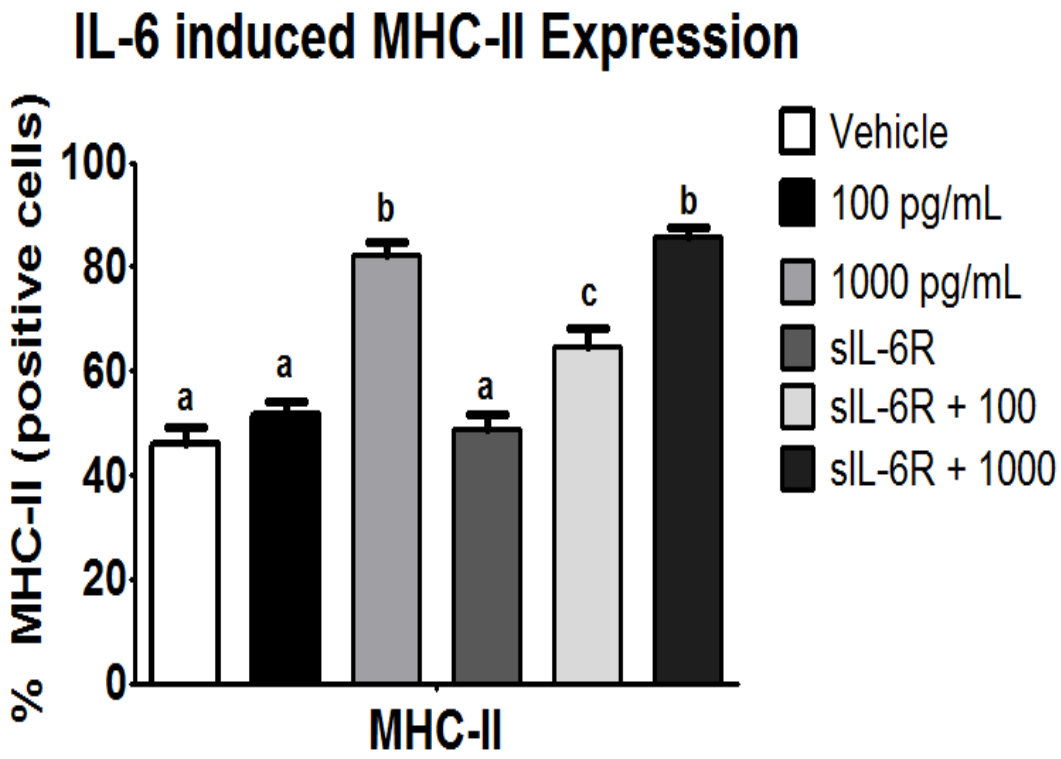


Figure 3.4: Differential expression of IL-6-induced MHC-II on microglia. Average percentage of cells that were pre-treated 1 h with vehicle or sIL-6R then treated 24 h with 100 or 1000 pg/mL IL-6. Cell surface markers were assessed for MHC-II expression; compared with isotype and unstained controls. Bars represent the mean ± SEM. Results are an average of 3 independent experiments. Means with different letters are significantly different from one another ($P<0.05$).

Adult

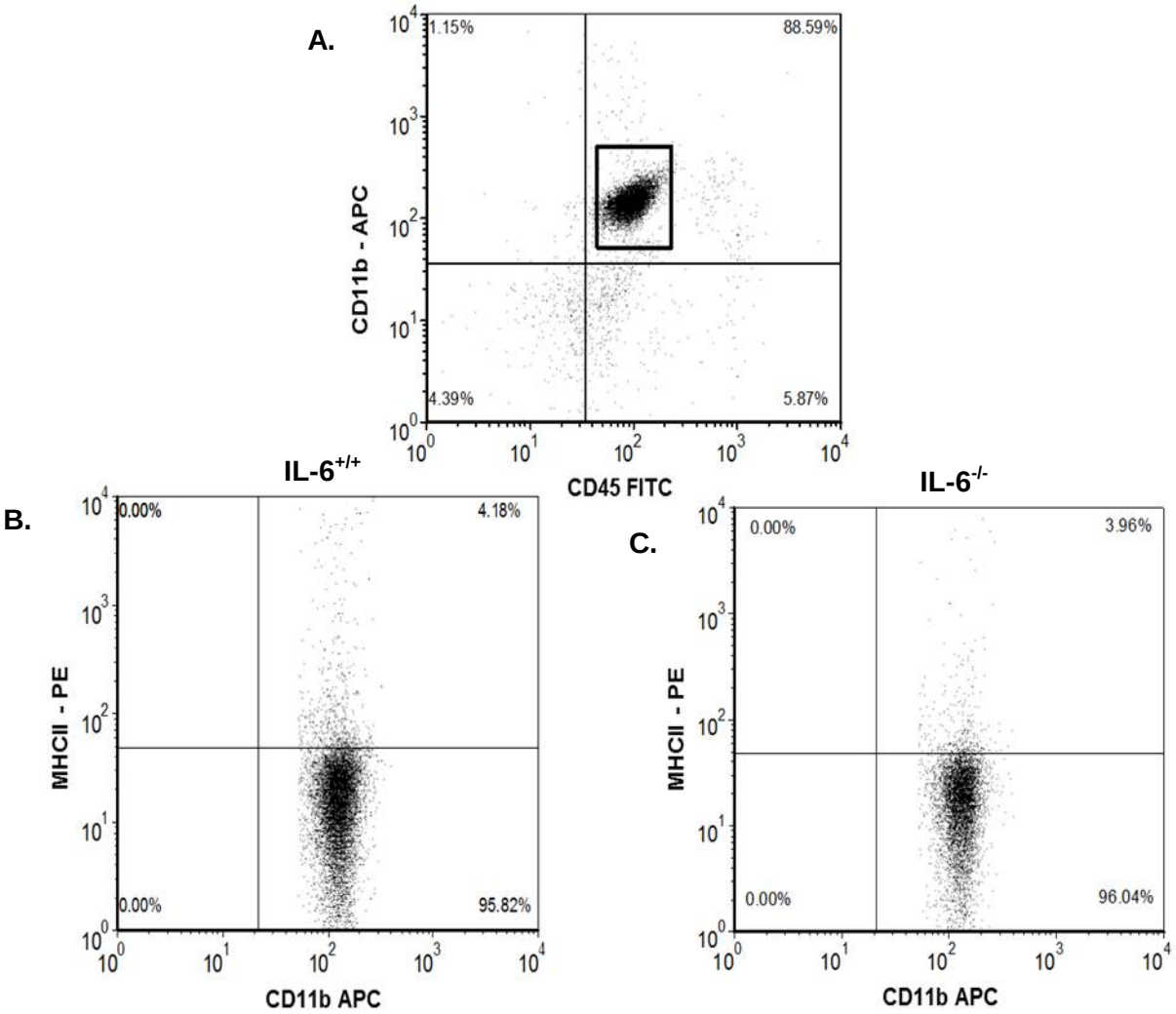


Figure 3.5: Expression of microglial MHC-II on adult IL-6$^{+/+}$ and IL-6$^{-/-}$ animals.
Representative two-color dot plot of percoll-isolated cells stained with anti-CD11b APC and anti-CD45 PE; identified by CD11b^{+}/CD45^{low} staining. Identified microglia were gated by CD11b/MHC-II positivity; expression of the cell surface markers were assessed and compared with isotype and unstained controls.

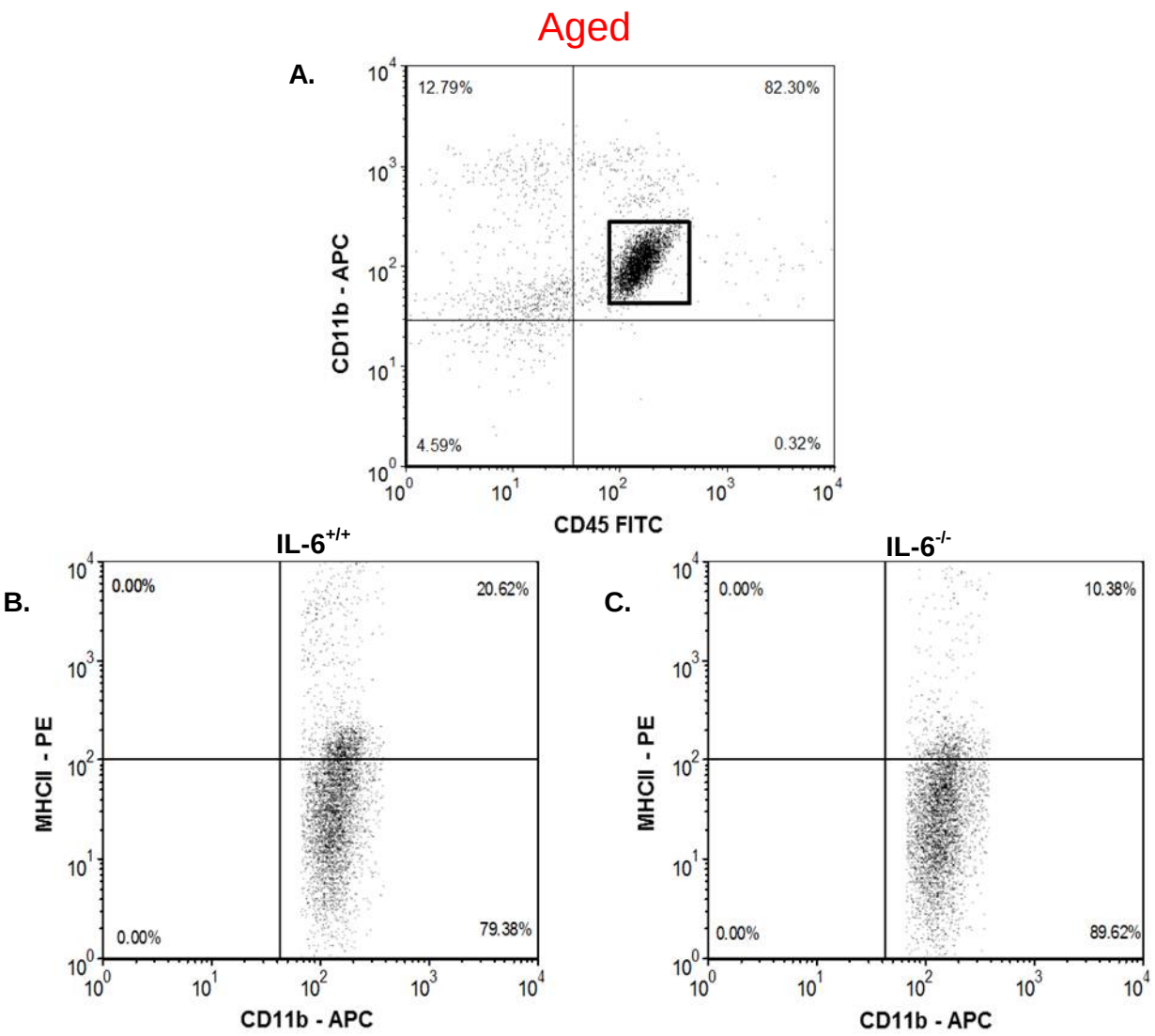


Figure 3.6: A reduction of microglial MHC-II on aged IL-6$^{-/-}$ compared to IL-6$^{+/+}$ animals. Representative two-color dot plot of percoll-isolated cells stained with anti-CD11b APC and anti-CD45 PE; identified by CD11b^{+}/CD45^{low} staining. Identified microglia were gated by CD11b/MHC-II positivity; expression of the cell surface markers were assessed and compared with isotype and unstained controls.

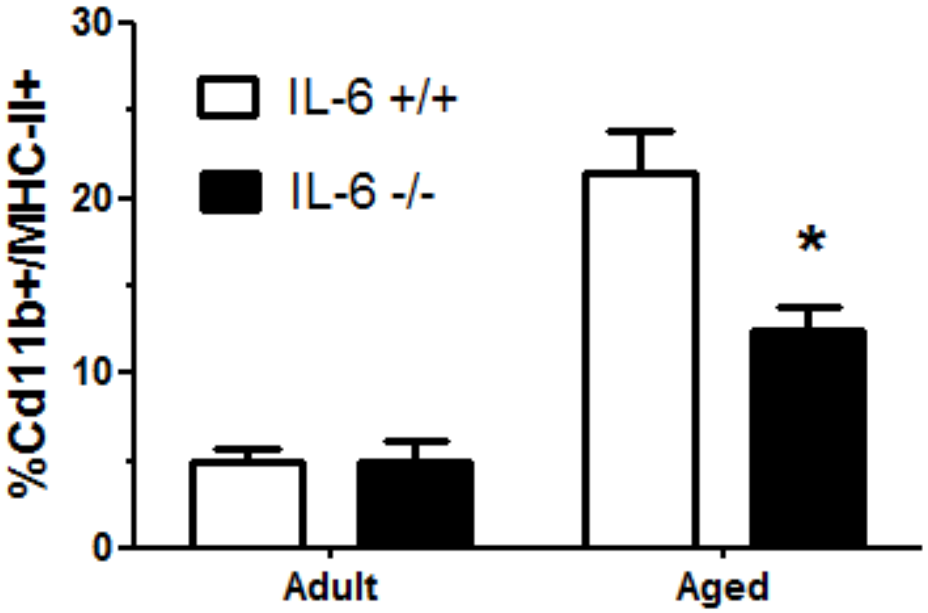


Figure 3.7: Differential expression of MHC-II on adult and aged IL-6$^{+/+}$ and IL-6$^{-/-}$ microglia. Average percentage of cells that were CD11b^{+}/MHC II. Cell surface markers were assessed for MHC-II expression; compared with isotype and unstained controls. Bars represent the means ± SEM (n = 6). Means marked with a * are significantly different from age-matched control ($P < 0.05$).

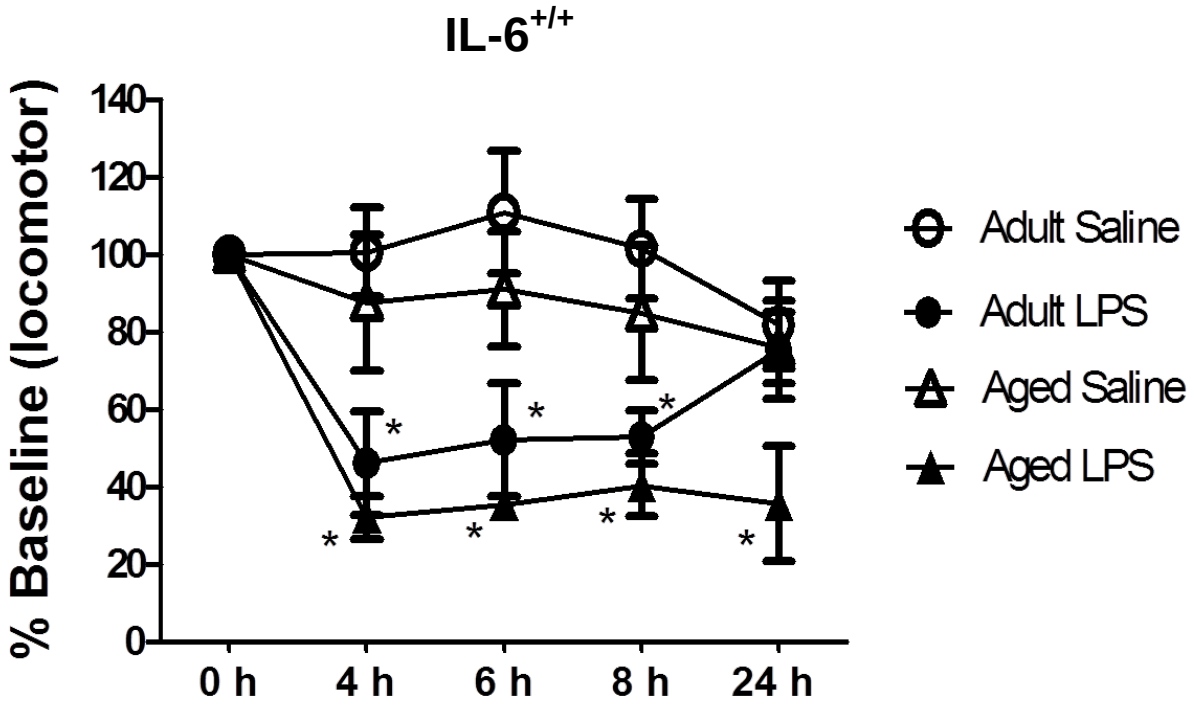


Figure 3.8: LPS-induced sickness behavior in adult and aged adult IL-6$^{+/+}$ animals. Mice were injected i.p. with sterile saline or LPS (100 µg). Spontaneous locomotor baseline behavior was measured before LPS injection (0) and at 4, 6, 8, and 24 h post-injection. Bars represent the mean ± SEM (n = 8-9) Means with * are statistically different (P<0.05) from saline controls.

.

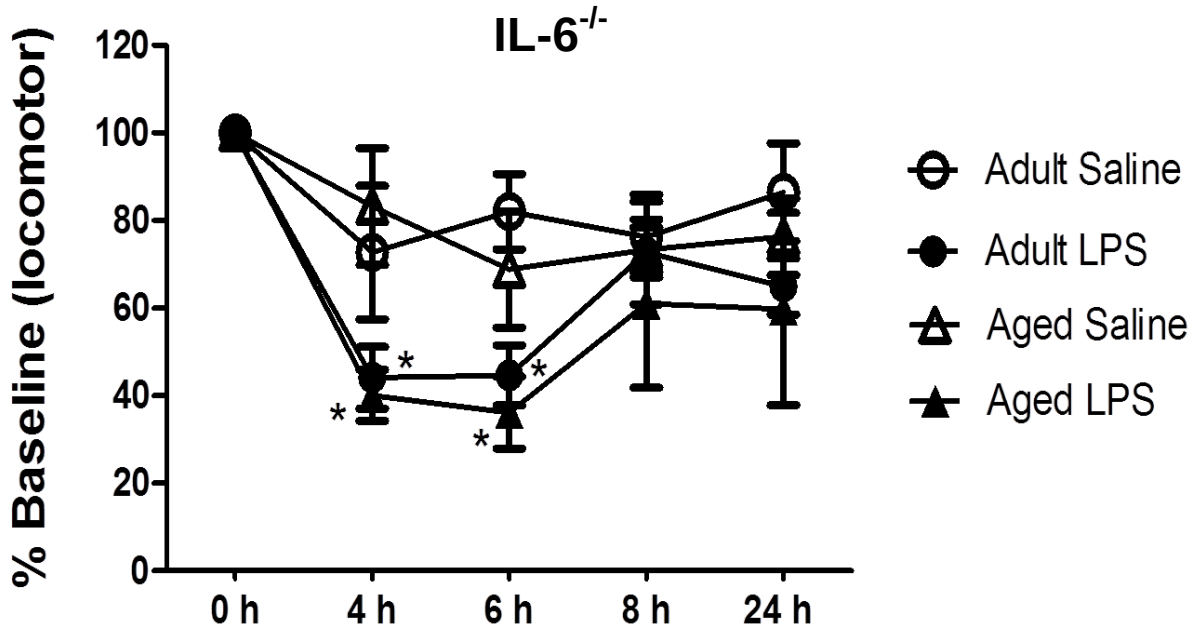


Figure 3.9: Adult and aged IL-6$^{-/-}$ mice recover more quickly from LPS-induced sickness behavior. Mice were injected i.p. with sterile saline or LPS (100 µg). Spontaneous locomotor baseline behavior was measured before LPS injection and at 4, 6, 8, and 24 h post-injection. Bars represent the mean ± SEM (n = 8-9) Means with * are statistically different (P<0.05) from saline controls.

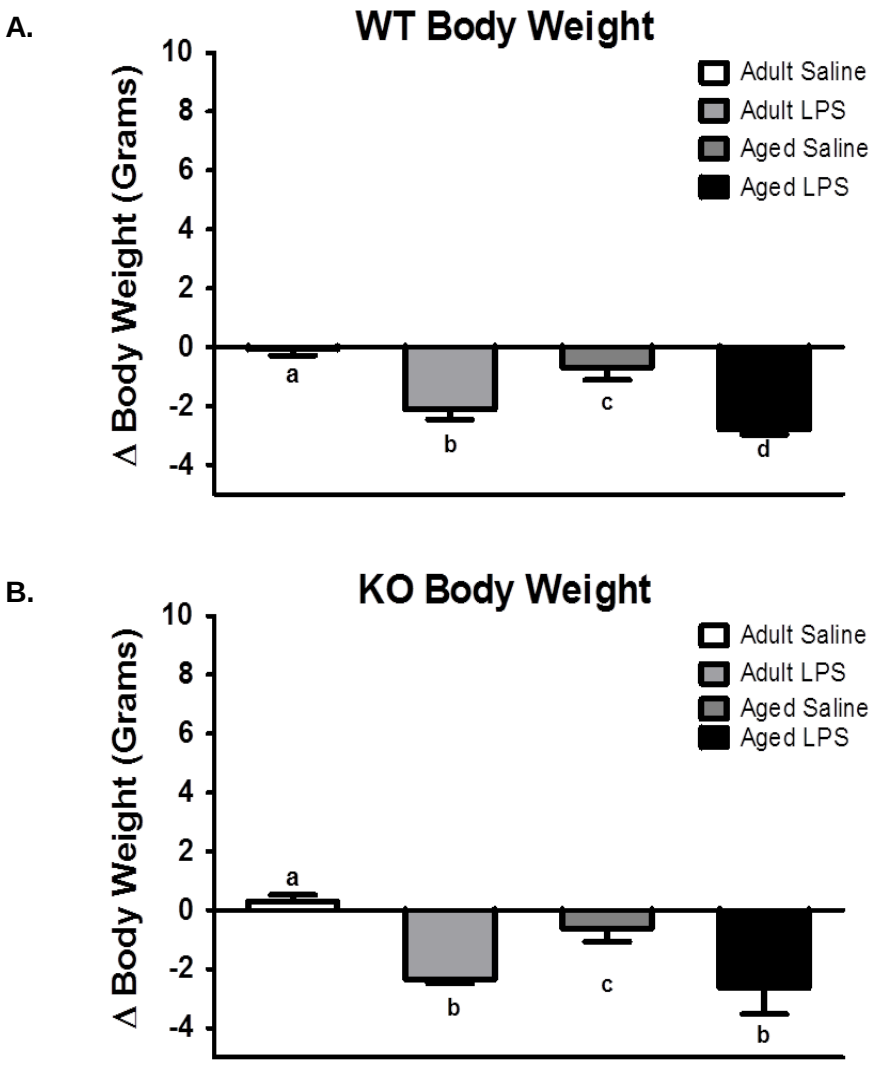
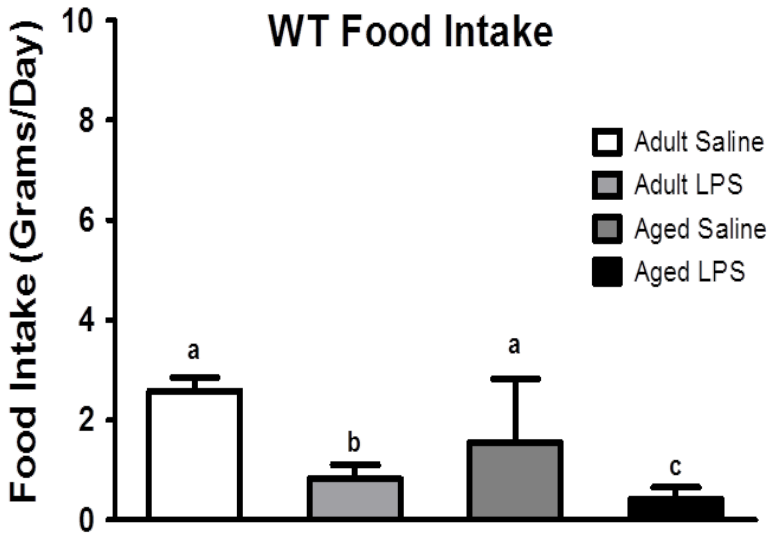


Figure 3.10: LPS-induced sickness behavior in adult and aged adult in IL-6$^{+/+}$ and IL-6$^{-/-}$ animals. Mice were injected i.p. with sterile saline or LPS (100 µg). Body weight was measured before LPS injection and at 24 h post-injection. Bars represent the mean ± SEM (n = 8-9) Means with different letters are statistically different (P<0.05) from saline controls.

A.



B.

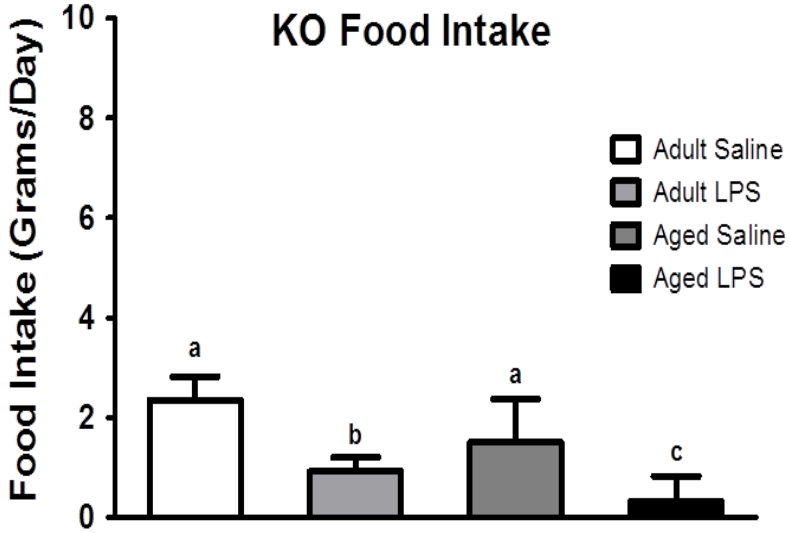


Figure 3.11: LPS-induced sickness behavior in adult and aged adult IL-6$^{+/+}$ and IL-6$^{-/-}$ animals. Mice were injected i.p. with sterile saline or LPS (100 µg). Food intake was measured before LPS injection and at 24 h post-injection. Bars represent the mean ± SEM (n = 8-9) Means with different letters are statistically different (P<0.05) from saline controls.

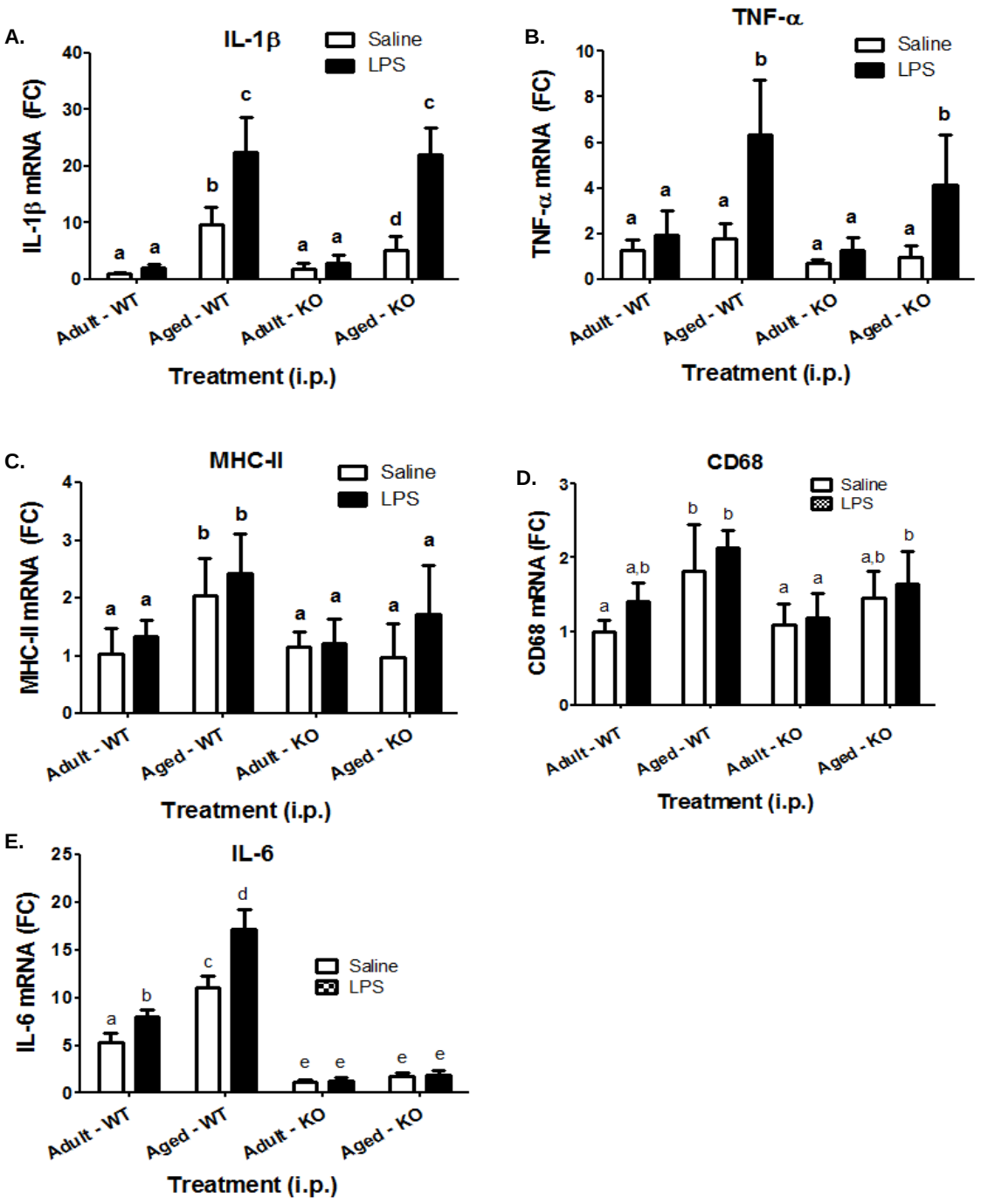


Figure 3.12: LPS-induced IL-6$^{+/+}$ and IL-6$^{-/-}$ gene expression. Hippocampal tissue was collected 24 h after i.p. LPS (100 µg) and assayed for IL-1β, TNF-α, MHC-II, CD68, and IL-6. Aged IL-6-/- animals had reduced baseline levels of IL-1β and MHC-II expression. Bars represent the mean ± SEM (n = 8-9) Means with different letters are statistically different from each other (P<0.05).

3.7 Literature cited

Abraham, J., Johnson, R.W., 2009. Central inhibition of interleukin-1 beta ameliorates sickness behavior in aged mice. Brain Behavior and Immunity 23, 396-401.

Banks, W.A., Kastin, A.J., Gutierrez, E.G., 1994. Penetration of Interleukin-6 across the murine blood-brain-barrier. Neuroscience Letters 179, 53-56.

Barrientos, R.M., Higgins, E.A., Biedenkapp, J.C., Sprunger, D.B., Wright-Hardesty, K.J., Watkins, L.R., Rudy, J.W., Maier, S.F., 2006. Peripheral infection and aging interact to impair hippocampal memory consolidation. Neurobiology of Aging 27, 723-732.

Burton, M.D., Johnson, R.W., 2011. Interleukin-6 trans-signaling in the senescent mouse brain is involved in infection-related deficits in contextual fear conditioning. Brain Behavior and Immunity 26, 738-738.

Burton, M.D., Sparkman, N.L., Johnson, R.W., 2011. Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. Journal of Neuroinflammation 8, 54.

Cahill, C.M., Rogers, J.T., 2008. Interleukin (IL) 1beta induction of IL-6 is mediated by a novel phosphatidylinositol 3-kinase-dependent AKT/IkappaB kinase alpha pathway targeting activator protein-1. Journal of Biological Chemistry 283, 25900-25912.

Campbell, I.L., Abraham, C.R., Masliah, E., Kemper, P., Inglis, J.D., Oldstone, M.B.A., Mucke, L., 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin-6. Proceedings of the National Academy of Sciences of the United States of America 90, 10061-10065.

Cardona, A.E., Huang, D., Sasse, M.E., Ransohoff, R.M., 2006. Isolation of murine microglial cells for RNA analysis or flow cytometry. Nature Protocols 1, 1947-1951.

Chen, J., Buchanan, J.B., Sparkman, N.L., Godbout, J.P., Freund, G.G., Johnson, R.W., 2008. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behavior and Immunity 22, 301-311.

Combrinck, M.I., Perry, V.H., Cunningham, C., 2002. Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease. Neuroscience 112, 7-11.

Cunningham, C., Campion, S., Lunnon, K., Murray, C.L., Woods, J.F., Deacon, R.M., Rawlins, J.N., Perry, V.H., 2009. Systemic inflammation induces acute behavioral and cognitive changes and accelerates neurodegenerative disease. Biological Psychiatry 65, 304-312.

Dantzer, R., Bluthe, R.M., Gheusi, G., Cremona, S., Laye, S., Parnet, P., Kelley, K.W., 1998. Molecular basis of sickness behavior. In: Kluger, M.J., Bartfai, T., Dinarello, C.A. (Eds.), Molecular Mechanisms of Fever, pp. 132-138.

Dilger, R.N., Johnson, R.W., 2008. Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. Journal of Leukocyte Biology 84, 932-939.

Ford, A.L., Goodsall, A.L., Hickey, W.F., Sedgwick, J.D., 1995. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. Journal of Immunology 154, 4309-4321.

Frank, M.G., Barrientos, R.A., Biedenkapp, J.C., Rudy, J.W., Watkins, L.R., Maier, S.F., 2006a. mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. Neurobiology of Aging 27, 717-722.

Frank, M.G., Wieseler-Frank, J.L., Watkins, L.R., Maier, S.F., 2006b. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. Journal of Neuroscience Methods 151, 121-130.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R., Samokhvalov, I.M., Merad, M., 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330, 841-845.

Godbout, J.P., Berg, B.M., Kelley, K.W., Johnson, R.W., 2004. alpha-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. Journal of Neuroimmunology 149, 101-109.

Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W., 2005. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. FASEB Journal 19, 1329.

Godbout, J.P., Johnson, R.W., 2004. Interleukin-6 in the aging brain. Journal of Neuroimmunology 147, 141-144.

Godbout, J.P., Johnson, R.W., 2009. Age and neuroinflammation: A lifetime of psychoneuroimmune consequences. Immunology and Allergy Clinics of North America 29, 321.

Godbout, J.P., Moreau, M., Lestage, J., Chen, J., Sparkman, N.L., Connor, J.O., Castanon, N., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system. Neuropsychopharmacology 33, 2341-2351.

Greenhill, C.J., Rose-John, S., Lissilaa, R., Ferlin, W., Ernst, M., Hertzog, P.J., Mansell, A., Jenkins, B.J., 2011. IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3. Journal of Immunology 186, 1199-1208.

Griffin, W.S., Mrak, R.E., 2002. Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. Journal of Leukocyte Biology 72, 233-238.

Gruol, D.L., Nelson, T.E., 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Molecular Neurobiology 15, 307-339.

Harden, L.M., du Plessis, I., Poole, S., Laburn, H.P., 2008. Interleukin (IL)-6 and IL-1 beta act synergistically within the brain to induce sickness behavior and fever in rats. Brain Behavior and Immunity 22, 838-849.

Henry, C.J., Huang, Y., Wynne, A., Hanke, M., Himler, J., Bailey, M.T., Sheridan, J.F., Godbout, J.P., 2008. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. Journal of Neuroinflammation 5.

Henry, C.J., Huang, Y., Wynne, A.M., Godbout, J.P., 2009. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1 beta and anti-inflammatory IL-10 cytokines. Brain Behavior and Immunity 23, 309-317.

Holmes, C., El-Okl, M., Williams, A.L., Cunningham, C., Wilcockson, D., Perry, V.H., 2003. Systemic infection, interleukin 1 beta, and cognitive decline in Alzheimer's disease. Journal of Neurology Neurosurgery and Psychiatry 74, 788-789.

Jang, S., Kelley, K.W., Johnson, R.W., 2008. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proceedings of the National Academy of Sciences of the United States of America 105, 7534-7539.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., Kohler, G., 1994. Imparied immune and acute-phase responses in interleukin-6 deficient mice. Nature 368, 339-342.

Krzyszton, C.P., Sparkman, N.L., Grant, R.W., Buchanan, J.B., Broussard, S.R., Woods, J., Johnson, R.W., 2008. Exacerbated fatigue and motor deficits in interleukin-10-deficient mice after peripheral immune stimulation. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 295, R1109-R1114.

Licastro, F., Grimaldi, L.M., Bonafe, M., Martina, C., Olivieri, F., Cavallone, L., Giovanietti, S., Masliah, E., Franceschi, C., 2003. Interleukin-6 gene alleles affect the risk of Alzheimer's disease and levels of the cytokine in blood and brain. Neurobiolgy of Aging 24, 921-926.

Maier, S.F., Goehler, L.E., Fleshner, M., Watkins, L.R., 1998. The role of the vagus nerve in cytokine-to-brain communication. Neuroimmunomodulation 840, 289-300.

Muller, T., Blum-Degen, D., Przuntek, H., Kuhn, W., 1998. Interleukin-6 levels in cerebrospinal fluid inversely correlate to severity of Parkinson's disease. Acta Neurologica Scandinavica 98, 142-144.

Nguyen, K., D'Mello, C., Le, T., Urbanski, S., Swain, M.G., 2011. Regulatory T cells suppress sickness behaviour development without altering liver injury in cholestatic mice. Journal of Hepatology.

Nguyen, M.D., D'Aigle, T., Gowing, G., Julien, J.P., Rivest, S., 2004. Exacerbation of motor neuron disease by chronic stimulation of innate immunity in a mouse model of amyotrophic lateral sclerosis. Journal of Neuroscience 24, 1340-1349.

Nguyen, M.D., Julien, J.P., Rivest, S., 2002. Innate immunity: The missing link in neuroprotection and neurodegeneration? Nature Reviews Neuroscience 3, 216-227.

Nimmerjahn, A., Kirchhoff, F., Helmchen, F., 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308, 1314-1318.

Njie, E.G., Boelen, E., Stassen, F.R., Steinbusch, H.W., Borchelt, D.R., Streit, W.J., 2012. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. Neurobiology of Aging 33, 195 e191-112.

Ogura, K., Ogawa, M., Yoshida, M., 1994. Effects of aging on microglia in the normal rat-brain - Immunohistochemical observations. Neuroreport 5, 1224-1226.

Penkowa, M., Moos, T., Carrasco, J., Hadberg, H., Molinero, A., Bluethmann, H., Hidalgo, J., 1999. Strongly compromised inflammatory response to brain injury in interleukin-6-deficient mice. Glia 25, 343-357.

Quan, N., 2008. Immune-to-brain signaling: How important are the blood-brain barrier-independent pathways? Molecular Neurobiology 37, 142-152.

Ransohoff, R.M., Perry, V.H., 2009. Microglial physiology: Unique stimuli, specialized responses. Annual Review of Immunology 27, 119-145.

Shafer, L.L., McNulty, J.A., Young, M.R.I., 2002. Brain activation of monocyte-lineage cells: Involvement of interleukin-6. Neuroimmunomodulation 10, 295-304.

Sparkman, N.L., Buchanan, J.B., Heyen, J.R.R., Chen, J., Beverly, J.L., Johnson, R.W., 2006. Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. Journal of Neuroscience 26, 10709-10716.

Vallieres, L., Lacroix, S., Rivest, S., 1997. Influence of interleukin-6 on neural activity and transcription of the gene encoding corticotrophin-releasing factor in the rat brain: An effect depending upon the route of administration. European Journal of Neuroscience 9, 1461-1472.

Vassiliadis, S., Papadopoulos, G.K., 1995. IL-6-mediated MHC class-II induction on RIN-5AH Insulinoma cells by IFN-gamma occurs via the G-protein pathway. Mediators of Inflammation 4, 374-379.

Wong, A.M., Patel, N.V., Patel, N.K., Wei, M., Morgan, T.E., de Beer, M.C., de Villiers, W.J., Finch, C.E., 2005. Macrosialin increases during normal brain aging are attenuated by caloric restriction. Neuroscience Letters 390, 76-80.

Ye, S.M., Johnson, R.W., 1999. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Zorina, Y., Iyengar, R., Bromberg, K.D., 2010. Cannabinoid 1 receptor and interleukin-6 receptor together induce integration of protein kinase and transcription factor signaling to trigger neurite outgrowth. Journal of Biological Chemistry 285, 1358-1370.

Chapter 4

Inhibition of interleukin-6 *trans*-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior

4.1 Abstract

Interleukin (IL)-6 is produced in the brain during peripheral infection and plays an important but poorly understood role in sickness behavior. Therefore, this study investigated the capacity of soluble gp130 (sgp130), a natural inhibitor of the IL-6 *trans*-signaling pathway to regulate IL-6 production in microglia and neurons *in vitro* and its effects on lipopolysaccharide (LPS)-induced sickness behavior *in vivo*. Murine microglia (BV.2) and neuronal cell lines (Neuro.2A) were used to study the effects of stimulating and inhibiting the IL-6 signaling pathway *in vitro*. *In vivo*, adult (3-6mo) BALB/c mice received an intracerebroventricular (icv) injection of sgp130 followed by an intraperitoneal (i.p.) injection of LPS, and sickness behavior and markers of neuroinflammation were measured. Soluble gp130 attenuated IL-6- and LPS-stimulated IL-6 receptor (IL-6R) activation along with IL-6 protein release in both microglial (BV.2) and neuronal (Neuro.2A) cell types *in vitro*. Moreover, *in vivo* experiments showed that sgp130 facilitated recovery from LPS-induced sickness, and this sgp130-associated recovery was paralleled by reduced IL-6 receptor signaling, mRNA, and protein levels of IL-6 in the hippocampus. Taken together, the results show that sgp130 may exert an anti-inflammatory effect on microglia and neurons by inhibiting IL-6 binding. These data indicate that sgp130 inhibits the LPS-induced IL-6 *trans*-signal and show IL-6 and its receptor are involved in maintaining sickness behavior.

4.2 Introduction

Peripheral infection stimulates production of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). These cytokines use neural and humoral pathways to convey a message to the brain (Maier et al., 1998; Quan, 2008). In the brain, the peripheral pro-inflammatory signal is mimicked by microglia, (Ransohoff and Perry, 2009) and the resulting cytokines target neurons to elicit sickness- related behaviors that are typically adaptive (Kelley et al., 2003). However, excessive cytokine production by microglia is associated with prolonged sickness behavior (Abraham and Johnson, 2009a; Combrinck et al., 2002; Godbout et al., 2005b; Huang et al., 2008), cognitive deficits (Campbell et al., 1993; Chen et al., 2008; Heyser et al., 1997), and affective disorders like anxiety and depression (Mines et al., 2010; Sparkman and Johnson, 2008). A recent study showed IL-6 knockout mice were refractory to LPS-induced increases of cytokines in the brain and cognitive deficits eluding to the potential permissive effects of IL-6 during LPS-induced sickness (Sparkman et al., 2006).

The IL-6 receptor is activated through two separate, but related pathways; classical- and *trans*-signaling. Classical IL-6 receptor activation is facilitated through the IL-6 ligand binding to its membrane-bound receptor. The receptor consists of two subunits: the IL-6 receptor-alpha chain (IL-6R), which binds IL-6, and the *trans*-membrane signaling subunit, glycoprotein 130 (gp130), which is the intra-cellular signal *trans*-ducer and is ubiquitously expressed. Both IL-6R and gp130 are cleaved immediately before the membrane spanning region by alternative splicing or shed by proteolytic enzymes to produce a soluble receptor located in extra-cellular matrix. It is

important to note that the expression pattern of IL-6R is limited to few cells of the immune system and conservatively dispersed among other cell types, meaning classical signaling is highly conserved. In contrast, gp130 is ubiquitously expressed (Heinrich et al., 1998; Kishimoto et al., 1992). The basis of *trans*-signaling is soluble IL-6R (sIL-6R) binding to IL-6 in the extra cellular matrix to form a IL-6/sIL-6R complex, which has an increased binding affinity to membrane-bound gp130 subunits, resulting in the capability of IL-6 production in any cell type that expresses gp130 (Jones et al., 2005; Rosejohn and Heinrich, 1994).

Upon binding through either the classical or *trans*-signal, gp130 dimerizes and autophosphorylates, resulting in the activation of Janus kinase-1 and 2 (Jak1 & Jak2). These tyrosine kinases phosphorylate the cytoplasmic region of gp130 creating recruitment sites for signal transducer and activation of transcription-3 (STAT3), a Src-homology-2 (SH2) domain-containing signaling molecule. Activated STAT3 forms a dimer, autophosphorylates, and translocates to the nucleus where it binds to enhancer elements of the IL-6 promoter region. Thus the main consequence of both classical or *trans*-signal IL-6 receptor action is to induce gene transcription and subsequent synthesis and secretion of IL-6, though *trans*-signaling allows this in many more cell types, due to the ubiquitous expression of gp130 (Heinrich et al., 1998). sIL-6R and soluble gp130 (sgp130) have varying effects on circulating IL-6. Where sIL-6R acts as an agonist, sgp130 acts as a partial antagonist, or decoy receptor, by binding IL-6 or the IL-6/sIL-6R complex and prevents the binding of membrane-bound gp130 and further signal transduction (Jostock et al., 2001).

The action of IL-6 is heavily dependent on the location of the receptors and the cell types exposed to the cytokine. For instance, IL-6 binding to IL-6R located on T-cells leads to the differentiation of stem line T-cells to helper T cells (Diehl and Rincon, 2002) whereas in the gastro-intestinal tract, IL-6 and its receptors on epithelial cells contribute to peripheral disorders such as colitis and Crohn's disease (Mudter and Neurath, 2007). However, studies examining IL-6 receptor signaling or *trans*-signaling in the CNS are limited and we are aware of no studies examining the extent to which IL-6 receptor signaling affects neuroinflammation and infection-related changes in behavior.

Therefore the purpose of this study was to assess classical and *trans*-signaling in neurons and microglia and determine if inhibiting IL-6 receptor signaling in the brain is sufficient to inhibit sickness behavior caused by peripheral infection. The important results showed treatment with sgp130 attenuated LPS-induced receptor activation and production of IL-6 and enhanced recovery of sickness behavior. These findings suggest that inhibition of excessive production of IL-6 through its signaling pathways during infection may be helpful in preventing behavioral deficits.

4.3 Materials and methods

BV.2 microglial and Neuro.2A neuronal cell culture

The murine microglia cell line, BV.2 (a gift from Linda Van Eldik, Northwestern University, Evanston, IL) and neuronal Neuro.2A cells (purchased from ATCC) have been used as a model to investigate the neuroimmune system (Jang et al., 2008; Zorina et al., 2010). Cells were maintained in 150-cm^2 tissue culture flasks (BD Falcon, Franklin Lakes, NJ) in DMEM (Bio-Whittaker, Cambrex, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 200 mM glutamine, and 100 units/ml penicillin/streptomycin

(Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator under 5% CO_2. Confluent cultures were passed by trypsinization. Cells were centrifuged (5 minutes (min) at 27°C, 200 × *g*), and culture medium was removed. In all experiments, cells were re-suspended in DMEM supplemented with 10% FBS and seeded in six-well plates (BD Falcon, Franklin Lakes,NJ) at a population of 3×10^5 - 5×10^5 cells per well overnight at 37°C in a humidified incubator under 5% CO_2 before treatments. Cells were treated with sterile saline containing 0.1% BSA (vehicle), sIL-6R, or sgp130 (R&D systems, Minneapolis, MN) for 1 hour (h) followed by treatment with recombinant IL-6 (R&D systems, Minneapolis, MN) or *Escherichia coli* LPS (serotype 0127:B8 Sigma, St. Louis, MO), for 20 min or 3 h, respectively.

Flow cytometry

Flow cytometric analysis of microglial and neuronal cell surface markers was performed as described previously, with a few modifications (Henry et al., 2008). In brief, Fc receptors on BV.2 microglia cells were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA) in a PBS-1% BSA/sodium azide solution, and incubated with anti-CD126-Phycoerythrin (PE) and anti-CD130-Allophycocyanin (APC) or anti-TLR-4 PE (eBiosciences, San Diego, CA), fluorescently labeled isotype antibodies for PE and APC (eBiosciences, San Diego, CA) were used for controls. Expression of surface receptors was determined using a Becton-Dickinson LSR II Flow Cytometer (Red Oaks, CA). Fifty thousand events were collected and flow data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Animals and surgery

Adult (3-6 months) male BALB/c mice obtained from our in-house breeding colony were used in all experiments. Mice were housed in polypropylene cages and maintained at 21°C under a reverse-phase 12 h light-dark cycle (lights off at 07:00) with *ad libitum* access to water and rodent chow.

Surgery: Intracerebroventricular (icv) cannulation was performed under aseptic conditions as described previously (Abraham et al., 2008). In brief, mice were deeply anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylazine (100 and 10 mg/kg, respectively) and the surgical site was shaved and sterilized. They were positioned in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA) so that the frontal and parietal bones of the skull were parallel to the surgical platform. An incision roughly 1.5 cm in length was made on the cranium to reveal the bregma and a 26-gauge stainless steel cannula (Plastics One, Roanoke, VA) was placed in the right lateral cerebral ventricle according to predetermined stereotaxic coordinates (lateral 1.6 mm and antero-posterior 1mm to the bregma, and horizontal 2 mm from the dura mater). The cannula was secured using two adjacent stainless steel screws and cranioplastic cement (Plastics One, Roanoke, VA). A dummy cannula (Plastics One, Roanoke, VA) was inserted in the guide cannula to prevent occlusion and infection. Mice were injected subcutaneously with buprenorphine (0.05 mg/kg) following surgery and then again 8-12 h later to aid with any post-operative discomfort. Mice were provided a minimum of seven days to recover from any discomfort or weight loss before any treatment or behavioral test. Accurate placement of the cannula was confirmed by allowing 2µl of sterile saline to flow via gravity into the lateral ventricle. If cannula

placement could not be confirmed, the animal was excluded from the study. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Animal studies

Mice were handled 1-2 min each day for seven days before experimentation to acclimate them to routine handling. On test day, animals were injected icv with sterile saline containing 0.1% BSA (vehicle) or 100 ng sgp130 dissolved in 2µl vehicle. At the same time as the icv injection, mice were injected i.p. with sterile saline or LPS (0.33 mg/kg BW or 10µg, serotype 0127:B8, Sigma, St. Louis, Mo.). The LPS dosage was selected because it elicits a proinflammatory cytokine response in the brain, which results in mild transient sickness behavior in adult mice (Berg et al., 2004). Tests were conducted during the dark phase (between 07:00 and 19:00) of the light/dark cycle under infrared lighting to aid video recording. Baseline behavior was taken just before treatment administration (0 h) and 4, 8, and 24 h afterwards.

To measure changes in cytokines and signaling molecules, mice not exposed to the behavior paradigms were injected icv with vehicle or sgp130 (100 ng) and i.p. with sterile saline or LPS (10 µg) and killed 8 h later by CO_2 asphyxiation. Blood samples were collected via cardiac puncture into EDTA-coated syringes to obtain plasma, and the brain was rapidly removed and dissected to obtain hippocampal tissue. Plasma and hippocampal tissue were snap-frozen in liquid nitrogen and stored at -80^{o}C until later analysis.

Behavioral tests

Social exploratory behavior: To assess motivation to engage in social exploration, a novel male juvenile conspecific (20-30 days old) from our in-house colony was introduced into the test subject's home cage for a 7 min period. Mice were video recorded, and the duration engaged in social investigation was determined from the video records by a trained observer who was blind to experimental treatments. Social behavior was determined as the amount of time that the experimental animal spent investigating (e.g. trailing, anogenital sniffing) the juvenile. Baseline social behavior was determined for all experimental treatments at the 0 h, for a 7 min period. Statistical analysis revealed there were no significant differences between treatment groups at baseline. The results are expressed as percent depression in time engaged in social behavior compared to respective baseline measures.

Western immunoblotting

To assess IL-6 receptor signaling in CNS cells *in vitro*, BV.2 and Neuro.2A cells were harvested and *in vivo,* mouse hippocampal tissue were allowed to thaw, and lysed in ice cold lysis buffer containing: 100 mM HEPES (7.5 pH), 150 mM NaCl, 1% Nonidet P-40 (U.S. Biological, Swampscott, MA), 2 mM EGTA, 2 mM Sodium Orthovanadate, Protease Inhibitor cocktail (100 mM EDTA, 1 µg/mL AEBSF, Bestatin, Pepstatin A, Leupeptin, Aprotinin, and E-64), and 1mM PMSF and centrifuged at 11000 × g for 10 min at 4^{o}C to remove all cellular debris. Protein concentration was determined using the BCA Protein Assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA). Lysate concentration was then normalized and denatured in SDS/PAGE buffer at 95^{o}C and stored at -20^{o}C until use. All lysates were electrophoresed and separated on a

7.5% SDS-PAGE gel, and transferred onto nitrocellulose membranes (GE Healthcare, Minneapolis, MN). The membranes were blocked with 5% non-fat milk and incubated with anti-phosphorylated STAT3 (tyr-705) antibody (Cell Signaling, Danvers, MA) overnight at 4°C. After incubation with an HRP-conjugated secondary antibody, the protein bands were detected with a chemiluminescenct substrate (Cell Signaling, Danvers, MA) and Bio-Max film (Eastman Kodak Company, Rochester, NY). For detection of total STAT3 protein, the membranes were stripped with stripping buffer (2% SDS, 6.25 mM Tris.HCL (6.8 pH), 0.70% β-ME) followed by overnight incubation with anti-STAT3 antibody (Cell Signaling, Danvers, MA) at 4°C. Immunoblot results were quantified using ImageJ 1.41 software (NIH).

Cytokine detection in cell supernatant, hippocampus, and plasma

Hippocampal tissue was lysed in ice cold lysis buffer and protein concentrations were determined using the BCA protein assay according to manufacturer's protocol. For hippocampal tissue, the antibodies and standards for the IL-6 ELISA were used according to the description by the manufacturer (eBiosciences San Diego, CA). Cell supernatants and plasma samples were assayed for IL-1β, TNF-α, IL-6, and the anti-inflammatory cytokine IL-10, using multiplexed bead-based immunoassay kits combined with a Cytokine Reagent kit as described by the manufacturer (Bio-Rad, Hercules, CA).

Cytokine mRNA measurement by quantitative real-time PCR

Total RNA from hippocampus was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO.) A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's protocol. Quantitative real time PCR was performed

using the Applied Biosystems (Foster, CA) Assay-on Demand Gene Expression protocol as previously described (Krzyszton et al., 2008). In brief, cDNA was amplified by PCR where a target cDNA (IL-6, Mm00446190_m1; IL-1β, Mm00434228_m1; TNF-α, Mm00443258_m1; IL-10, Mm00439616_m1, IL-6R, 00439653_m1; and gp130, mM00439665_m1) and a reference cDNA (glucose-3 phosphate dehydrogenase, Mm99999915_g1) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR reactions were performed in triplicate under the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, and results are expressed as fold difference.

Statistical analysis

All data were analyzed using the ANOVA routine in Statview and MIXED procedure of the Statistical Analysis System software (SAS Inst., Cary, NC). Data were subjected to a univariate analysis to ensure normality. Behavioral data were subjected to a three-way ANOVA (sgp130 × LPS × time) using repeated measures in which Time (0, 4, 8, and 24 h) was a within subjects measure, and sgp130 and LPS were between subjects measures. Cytokine mRNA and protein levels were analyzed using a two-way ANOVA (sgp130 × LPS). Phosphorylation of STAT3 levels were analyzed using a two-way ANOVA (sIL-6R or sgp130× LPS). Post hoc Student's *t* test of least square means was used to determine if treatment means were significantly different from one another ($p<0.05$). All data are presented as mean ± standard error of the mean (SEM).

4.4 Results

IL-6 and LPS induce STAT3 phosphorylation in microglia and neurons

To verify the presence of the subunits involved in IL-6 and LPS signaling, the cell surface expression of IL-6R, gp130, and TLR-4 on BV.2 and Neuro.2A cells was examined. Figure 4.1A shows more than 50% of the microglial BV.2 cells expressed gp130 while nearly 90% expressed IL-6R; approximately 50% of the BV.2 cells expressed both IL-6R and gp130. In contrast, about 90% of the Neuro.2A cells expressed gp130, 3% expressed IL-6R, and 3% co-expressed IL-6R and gp130. Approximately 80% of the BV.2 cells expressed TLR-4 compared to 30% of the Neuro.2A cells (Figure 4.1B). Although IL-6 can activate multiple transcription factors (e.g., STAT3, AP-1, CREB), in CNS cells activation of the IL-6 receptor upregulates STAT3 phosphorylation (Heinrich et al., 1998; Marz et al., 1999; Schobitz et al., 1995). Thus, the capacity of IL-6 to induce the phosphorylation of STAT3 in BV.2 and Neuro.2A cell cultures was examined. Figure 4.2 shows that IL-6 at a higher concentration (50 ng/mL) increased phosphorylated STAT3 similarly in microglia and neurons. However, at a lower concentration (10 ng/mL), IL-6 only increased STAT3 phosphorylation in microglia, which is consistent with the greater proportion of these cells that expressed IL-6R. These findings suggest that classic and *trans*-signaling can occur on both neurons and microglia, although neurons may be more readily regulated through the mechanism of *trans*-signaling since gp130 is highly expressed on this cell type.

IL-6 *trans*-signaling in microglia and neurons

Previous studies have shown that gp130 is expressed constitutively on all cell types (Dreuw et al., 2004; Taga and Kishimoto, 1997) and this expression facilitates *trans*-signaling in the presence of IL-6 and sIL-6R (Jones et al., 2005; Jones and Rose-John, 2002; Rosejohn and Heinrich, 1994). Figure 4.3A shows that pretreatment of microglia and neurons with sIL-6R significantly increased IL-6-induced STAT3 phosphorylation. Consistent with the increase in STAT3 phosphorylation, a sIL-6R × LPS interaction was evident whereby sIL-6R upregulated LPS-induced IL-6 production in microglia ($p<0.05$), and neurons ($p<0.04$, Figure 4.4). Although not statistically significant, there was some constitutive STAT3 phosphorylation and IL-6 expression in samples pretreated with sIL-6R (Figures 4.3 and 4.4).

sgp130 attenuated IL-6R activation in microglia and neurons

We next investigated the ability of sgp130 to alter phosphorylation of STAT3 and expression of IL-6. A sgp130 × LPS interaction revealed that pretreatment of BV.2 microglial ($p<0.03$) and Neuro.2A neuronal ($p<0.02$) cells with sgp130 decreased LPS-induced activation of STAT3 and inhibited LPS-induced IL-6 production in both BV.2 and Neuro.2A cells (Figures 4.5 and 4.6, respectively). These data demonstrate that sgp130 inhibits LPS-induced IL-6 production in microglia and neurons. The LPS-induced secretion of IL-1β, TNF-α, and IL-10 was not affected by sgp130 (data not shown).

sgp130 inhibits LPS-induced sickness behavior

Brain microglia and neurons produce inflammatory cytokines, including IL-6, that induce sickness behavior. Given the *in vitro* results, we investigated the effect of centrally administered sgp130 on LPS-induced sickness behavior. Social exploratory behavior was used to measure sickness. Three-way ANOVA of social behavior revealed a significant LPS × time × sgp130 interaction ($p<0.01$). As expected, LPS treatment decreased social exploratory behavior in a time-dependent manner. LPS induced transient sickness, as the behavior of mice given LPS returned to baseline by 24 h post-injection (Figure 4.7). However, behavior of mice treated icv with sgp130 prior to LPS returned to normal sooner. That sgp130 did not inhibit LPS-induced sickness behavior at 2 or 4 h after LPS treatment but did later, suggests the IL-6 *trans*-signaling pathway is important for maintaining sickness behavior but not for its induction.

sgp130 attenuated STAT3 phosphorylation and IL-6 gene expression and protein in the brain

Because sgp130 inhibited LPS-induced sickness behavior 8 h post-injection, hippocampal tissue and plasma was collected from a separate group of sgp130- and LPS-treated mice at the 8 h time point to assess STAT3 phosphorylation and IL-6 expression. Similar to the *in vitro* results, i.p. LPS upregulated STAT3 phospho-protein in the hippocampus. There was a sgp130 × LPS interaction ($p<0.001$) whereby STAT3 phosphorylation was blunted when mice were given icv sgp130 (Figure 4.8B). There was also a significant sgp130 × LPS interaction ($p<0.02$) whereby sgp130 decreased the amount of LPS-induced IL-6 mRNA in the hippocampus, although it did not significantly affect IL-1β or TNF-α mRNA (Figure 4.6B). To determine if the effect of

sgp130 was also apparent at the protein level, LPS-induced IL-6 protein was measured. As expected, LPS alone increased IL-6 protein in the hippocampus; however, a sgp130 × LPS interaction ($p<0.0001$) indicated that co-administration of sgp130 inhibited the LPS-induced increase in IL-6 (Figure 4.9B). Taken together, these results show that sgp130-related changes in LPS-induced social behavior are paralleled by sgp130-associated changes in the brain.

To assess the effect of icv sgp130 on the peripheral cytokine response to i.p. LPS, plasma was assayed for IL-1β, IL-6, IL-10, and TNF-α. Plasma levels of all four cytokines was increased after LPS treatment and this was not affected by sgp130 (Table 1), suggesting icv sgp130 acts locally in the brain.

4.5 Discussion

Bi-directional communication between the periphery and the brain is important for the appropriate response to an immune stimulus (Dantzer et al., 1998). During peripheral infection, pro-inflammatory cytokines are produced in the brain and play a role in adaptive sickness behavior. However, an excessive cytokine response in the brain is associated with prolonged sickness behavior (Abraham and Johnson, 2009a; Combrinck et al., 2002; Godbout et al., 2005b; Huang et al., 2008), cognitive deficits (Campbell et al., 1993; Chen et al., 2008; Heyser et al., 1997), and increased anxiety (Mines et al., 2010; Sparkman and Johnson, 2008); and the specific role of IL-6 has not been extensively studied. We investigated the capacity of sgp130 to block IL-6 production in microglia and neurons *in vitro* and explored the effects of sgp130 on LPS-induced sickness behavior *in vivo*. *In vitro*, sgp130 attenuated LPS-stimulated IL-6R activation along with IL-6 protein release in both microglial (BV.2) and neuronal

(Neuro.2A) cell types. Moreover, *in vivo* experiments showed that icv sgp130 facilitated recovery from LPS-induced sickness, and this sgp130-associated recovery was paralleled by reduced IL-6 mRNA and protein levels in the hippocampus.

The present study demonstrates that murine microglia and neurons have the potential to produce IL-6 through both a classic and *trans*-signaling pathway. In two-color flow cytometry experiments, we showed that BV.2 and Neuro.2A cells expressed both gp130 and IL-6R on their cell surface, though expression differed in each cell type. The findings indicate that classic and *trans*-signaling are important on both neurons and microglia, though neurons may be more readily regulated through the mechanism of *trans*-signaling. Previous studies report that the presence of the sIL-6R elicits an exaggerated production of IL-6 protein (Jones and Rose-John, 2002; Mizuguchi et al., 2001; Nancey et al., 2008). Consistent with these reports, we found that pretreatment of sIL-6R led to an IL-6- and LPS-induced increase of IL-6 protein in microglia and neurons. This response is presumably elicited by the ligand and soluble receptor forming a sIL-6R/IL-6 complex. This complex has the ability to bind to the gp130 *trans*-membrane receptor signal transducer and activate intracellular signals that produce IL-6 in any cell type via this *trans*-signaling mechanism.

LPS binds TLR-4, which we confirmed was present on both microglia and neurons (Figure 4.1C and D). Upon binding, LPS induces upregulation of the NF-κB transcription factor that binds promoter regions to stimulate the production of IL-6 along with a milieu of other cytokines (e.g. IL-1β, TNF-α, and IL-10) (Laflamme and Rivest, 2001). Soluble gp130 inhibits IL-6 *trans*-signaling but also regulates IL-6 related cytokines oncostatin M (OSM) and leukemia inhibitory factor (LIF). However, sgp130

has a much lower affinity for OSM and LIF than for the IL-6/sIL-6R complex (Jostock et al., 2001) and would not be expected to affect either cytokine at the dose used here (Jostock et al., 2001; Modrell et al., 1994). Therefore, using sgp130 allowed us to investigate the effects of IL-6 after LPS treatment, while leaving all other cytokines unaffected.

Successful activation of the IL-6R is noted by the dimerization of gp130, resulting in an intracellular cascade that forms recruitment sites for STAT3 in the cytoplasmic region. STAT3 homodimerizes, autophosphorylates, then translocates to the nucleus and binds to enhancer elements of IL-6 to induce gene transcription. Here, STAT3 was upregulated in response to both IL-6 and LPS in BV.2 and Neuro.2A cells and pretreatment with sIL-6R led to an increased IL-6- and LPS-induced STAT3 phosphorylation. However, when pretreated with sgp130, IL-6 and LPS-stimulated BV.2 and Neuro.2A cells displayed a decrease in STAT3 phosphorylation. These data agree with other studies using sgp130 to inhibit IL-6 signaling in peripheral models of inflammation such as arthritis, peritonitis, and colitis (Coles et al., 2007; McLoughlin et al., 2005; Richards et al., 2006). To our knowledge, this is the first study to report that pretreatment with sgp130 attenuated LPS-induced IL-6 protein secretion in CNS-derived cells.

LPS activation of the peripheral innate immune system stimulates a robust secretion of inflammatory cytokines through the NF-κB pathway and these cytokines are relayed to the CNS via vagal nerve afferents, and humoral and diffusive pathways (Maier et al., 1998; Quan, 2008). Once in the brain this inflammatory signal is mimicked by innate immune cells (Block et al., 2007; Kreutzberg, 1996; Ransohoff and Perry,

2009) and targets neurons which elicit a sickness behavior response that includes general malaise, decreased activity, decreased social interaction, decreased food and water intake (weight loss), and sleep dysregulation (Dantzer et al., 1998; Kelley et al., 2003; Maier and Watkins, 1998). We therefore investigated the effects of icv sgp130 *in vivo* and hypothesized that, given the role of IL-6 in neuroinflammatory responses; it would attenuate LPS-induced sickness behavior and IL-6 production. Here we show that sgp130 was effective in facilitating the recovery from LPS-induced social exploratory behavior as early as 8 h in mice. In addition to facilitating recovery from LPS, sgp130 attenuated receptor activation, gene expression, and production of IL-6 in adult mice 8 h after LPS injection. Consistent with previous studies (Berg et al., 2004; Godbout et al., 2004; Godbout et al., 2005a; Henry et al., 2008), a reduction in brain cytokines did not prevent the initial induction of LPS-induced sickness behavior seen at 2-4 h post-injection, but rather facilitated the recovery from sickness starting at the 8 h time point (Jones and Rose-John, 2002; Mizuguchi et al., 2001; Nancey et al., 2008). In this model, the inability of sgp130 to block the onset of sickness behavior can be attributed to the fact that LPS induces multiple proinflammatory cytokines that have redundant properties and inhibition of a single cytokine is not sufficient to block the initial sickness. Notably, a study showed that LPS-induced sickness behavior was blocked only if IL-1β, IL-6, and TNF-α were antagonized simultaneously (Swiergiel and Dunn, 1999).

This facilitation in recovery from LPS-induced sickness has been observed in various nutritional and pharmacological interventions (Abraham and Johnson, 2009a, b; Berg et al., 2005; Richwine et al., 2005) and may be of particular importance when

considering conditions where an exaggerated response is elicited during a primed inflammatory state, such as in overexpressing transgenic animals (Campbell et al., 1993; Heyser et al., 1997), prion disease (Combrinck et al., 2002), and aging (Chen et al., 2008; Godbout et al., 2005b; Huang et al., 2008). We have previously demonstrated that aged animals display an exaggerated neuroinflammatory and sickness behavior response after activation of the peripheral immune system (Godbout et al., 2005b) and it appears that primed microglia are responsible for this exacerbated phenotype (Godbout et al., 2004; Sparkman and Johnson, 2008; Ye and Johnson, 1999). We and others have shown interventions that are anti-inflammatory are able to ameliorate the exaggerated cytokine response in the brain as well as the mal-adaptive behavioral response that results from peripheral infection (Abraham and Johnson, 2009a, b; Berg et al., 2005; Henry et al., 2008; Jang et al., 2010; Richwine et al., 2005). Based on the data obtained from this study, it is possible that sgp130 will abrogate the prolonged LPS-induced alterations in sickness behavior, cognition, as well as exaggerated IL-6 levels exhibited in aged mice.

Studies have highlighted the potential therapeutic role of sgp130 in treating inflammation; it has been shown to suppress the severity of experimentally-induced arthritis, modulate leukocyte trafficking, and mitigate the effects of colitis and colon cancer (Atreya et al., 2000; Chen et al., 2004; Fielding et al., 2008; Jones et al., 2005; Richards et al., 2006). The current study is the first to extend the body of literature and show the effectiveness of sgp130 in inhibiting IL-6 signaling in cells of the CNS and in brains of animals. The present results suggest that the use of sgp130 as an inhibitor of the IL-6 pathway in an array of inflammatory conditions, from arthritis to

neuroinflammatory disorders, may mitigate IL-6 expression and have a beneficial effect on behavioral responses.

4.6 Figures

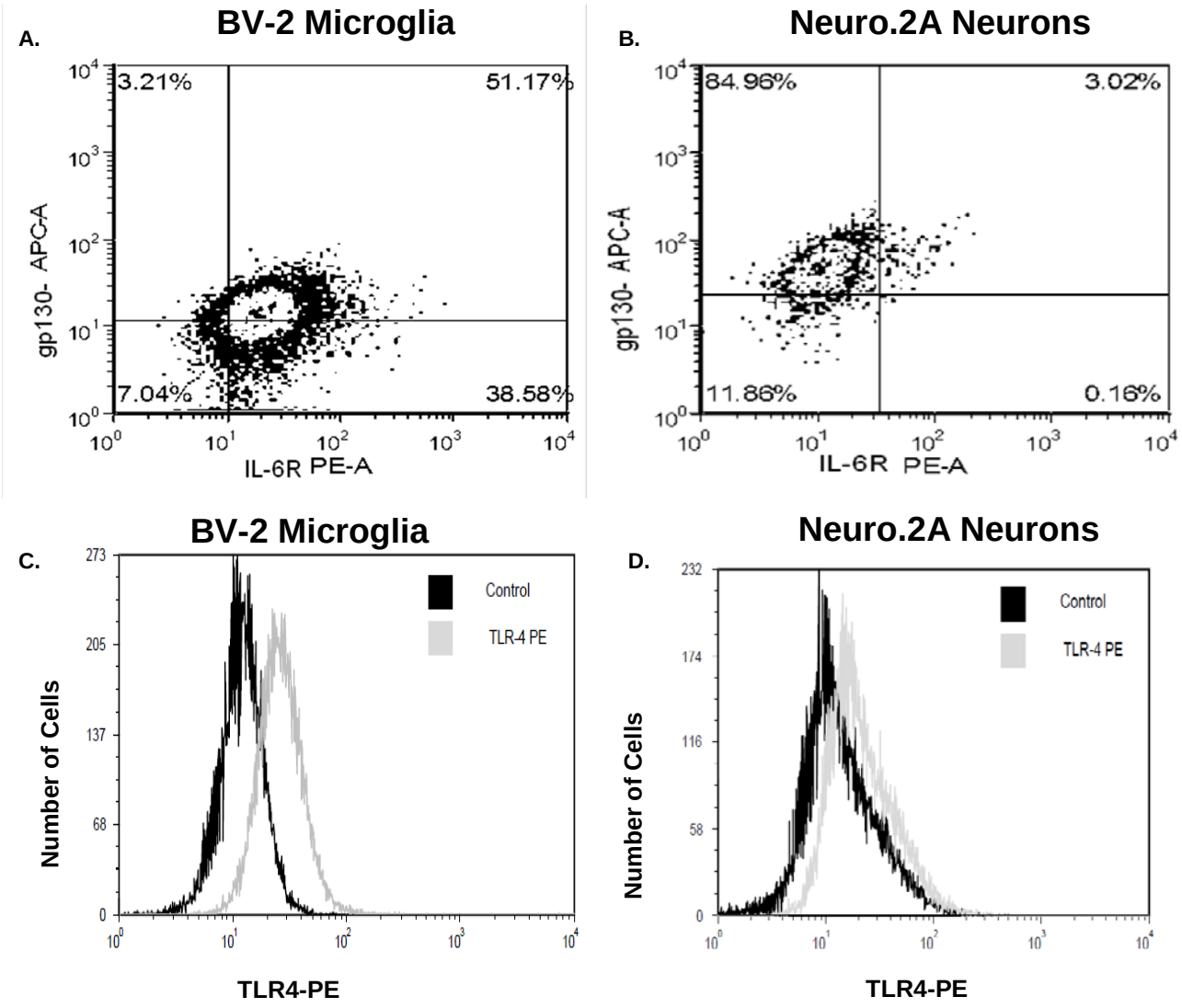


Figure 4.1: Differential expression of IL-6R, gp130, and TLR-4 on microglia and neurons. A and B) Two-color dot plot; cells were incubated with anti-CD126-PE and anti-CD130-APC and expression of the cell surface markers were assessed on microglia and neurons. C and D) Single-parameter histograms; cells were incubated with anti-TLR-4-PE and compared with isotype controls.

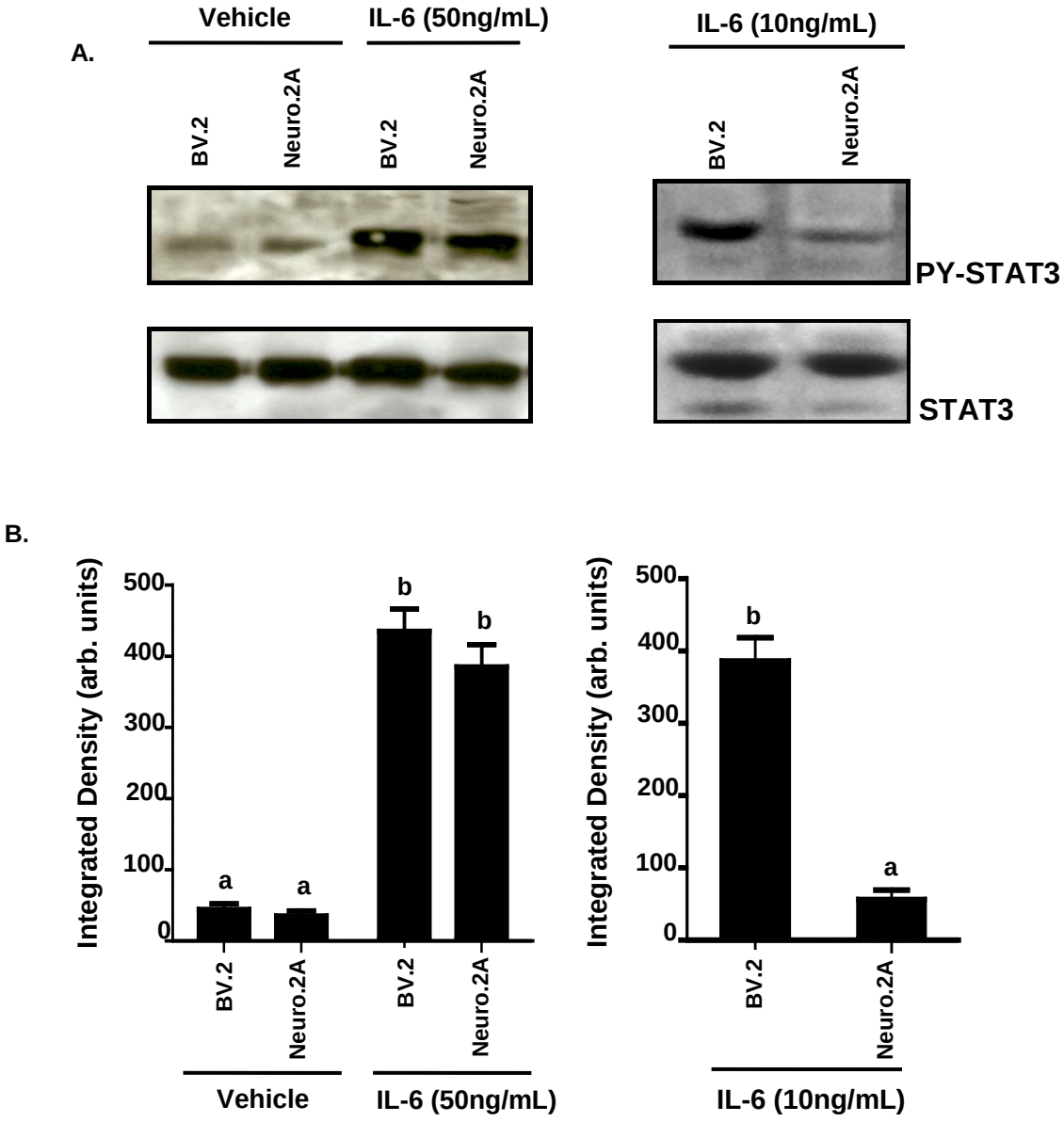


Figure 4.2: IL-6-induced STAT3 expression in BV.2 microglial and Neuro.2A neuronal cells. IL-6 receptor activation in BV.2 and Neuro.2A cells was verified by STAT3 phosphorylation 20 min after treatment with 10 and 50 ng/mL of IL-6. Bars represent the mean ± SEM. Results are an average of 5 independent experiments. Means with different letters are significantly different from one another ($p<0.05$).

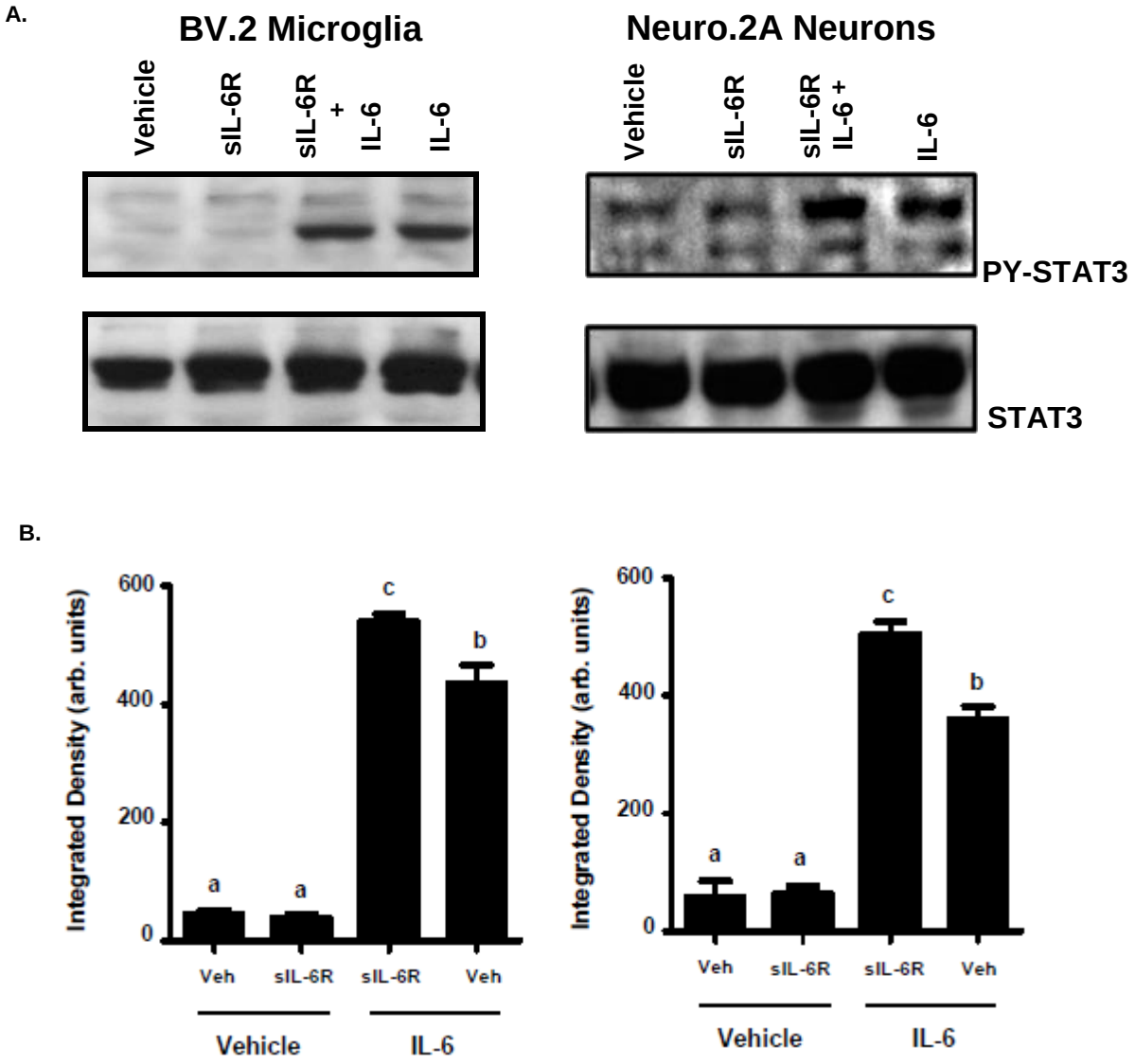


Figure 4.3: IL-6 *trans*-signaling in BV.2 microglia and Neuro.2A cells.
BV.2 and Neuro2A cells were pre-treated for 1 h with 25 ng/mL sIL-6R and A) IL-6-induced STAT3 phosphorylation. B) Bars represent the mean ± SEM. Results are an average of 5 independent experiments. Means with different letters are significantly different from one another ($p<0.05$).

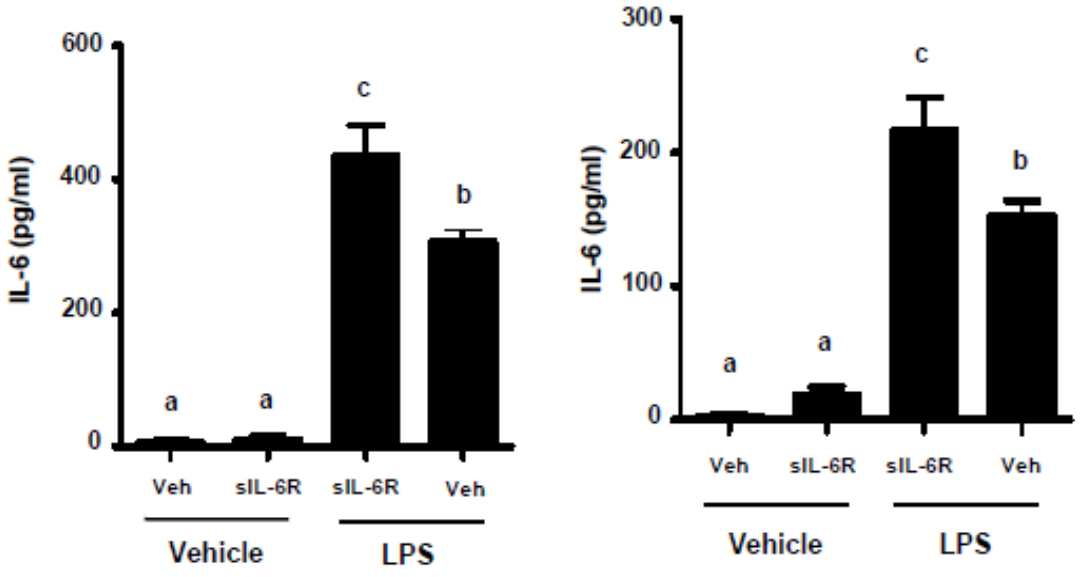


Figure 4.4: IL-6 *trans*-signaling in BV.2 microglia and Neuro.2A cells. LPS-induced IL-6 protein secretion was measured at 3 h. Bars represent the mean ± SEM. Results are an average of 5 independent experiments. Means with different letters are significantly different from one another ($p<0.05$).

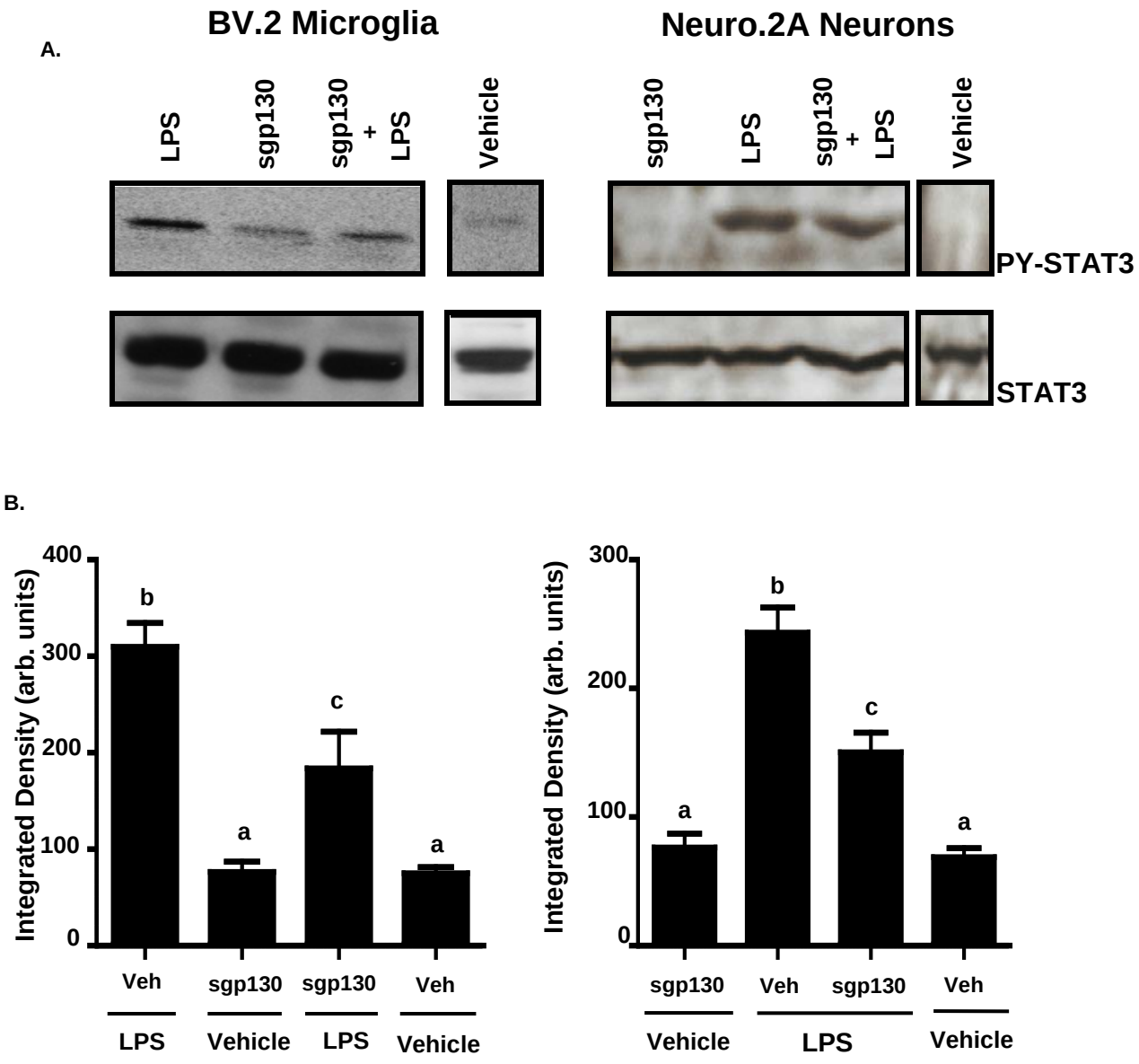


Figure 4.5: sgp130 attenuation of LPS-induced STAT3 phosphorylation in Neuro.2A and BV.2 cells. BV.2 microglia and Neuro.2A neuronal cells were pre-treated for 1 h with 100 ng sgp130 and A) LPS-induced STAT3 phosphorylation was measured. B) Bars represent the mean ± SEM. Results are an average of 5 independent experiments. Means with different letters are significantly different from each other ($p<0.05$).

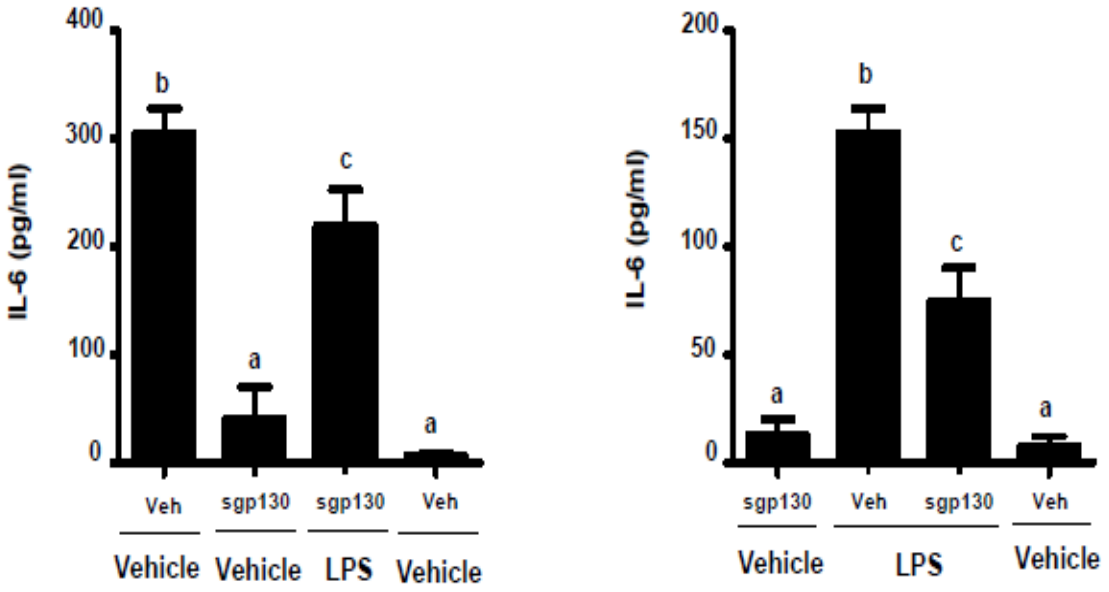


Figure 4.6: sgp130 attenuation of LPS-induced STAT3 phosphorylation in Neuro.2A and BV.2 cells. IL-6 protein secretion was measured. Bars represent the mean ± SEM. Results are an average of 5 independent experiments. Means with different letters are significantly different from each other ($p<0.05$).

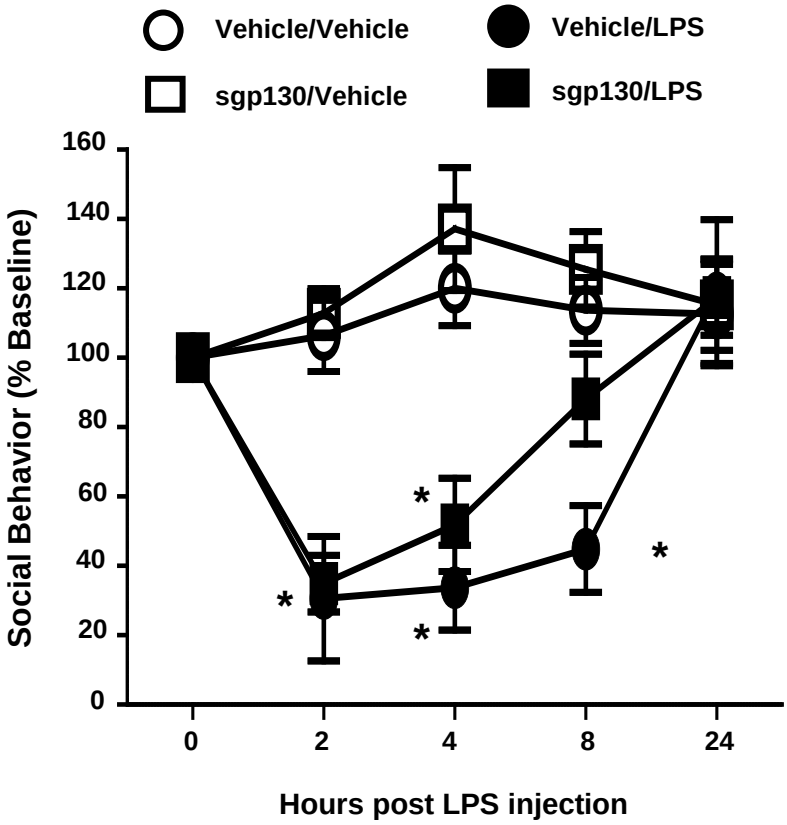


Figure 4.7: sgp130 facilitates recovery from LPS-induced sickness behavior. Mice were injected icv with vehicle or 100 ng sgp130 and i.p. with sterile saline or LPS. Social exploratory baseline behavior was measured before LPS injection and at 2, 4, 6, 8, and 24 h post-injection. Bars represent the mean ± SEM (n = 11-12) Means with * are statistically different ($p<0.05$) from saline controls.

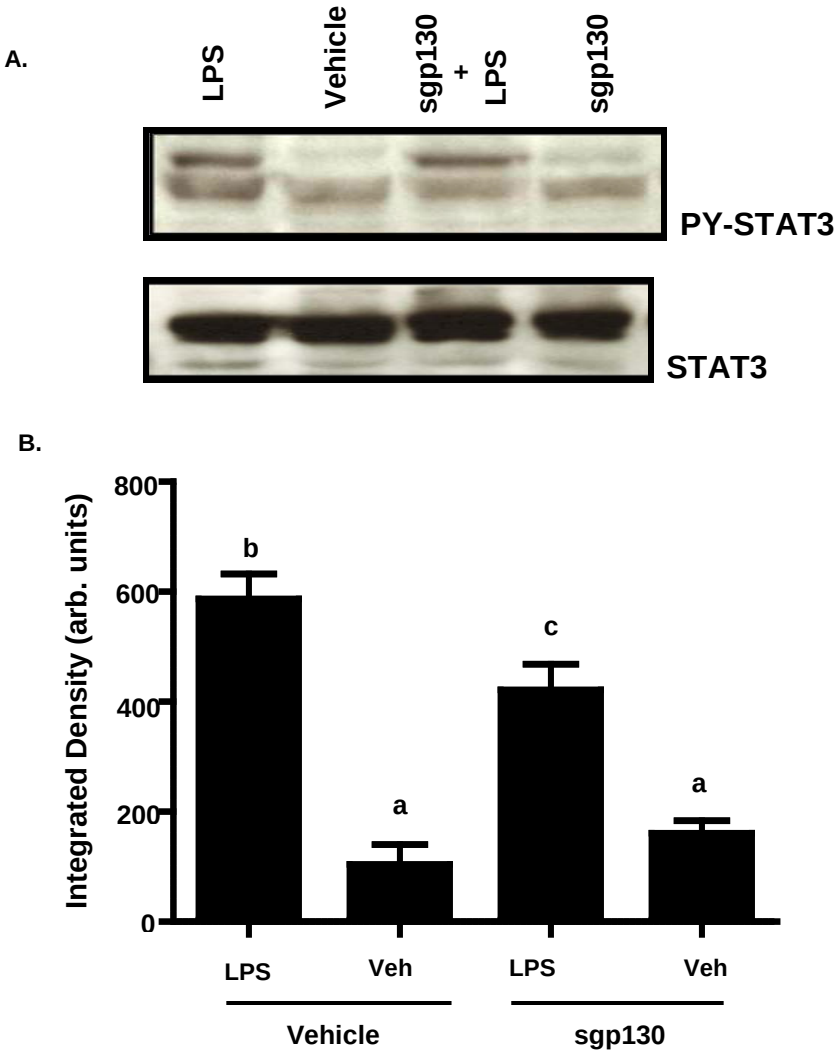


Figure 4.8: sgp130 reduces IL-6 receptor activation, gene expression, and protein secretion *in vivo*. Hippocampal tissue was collected 8 h after icv sgp130 and i.p. LPS and assayed for STAT3 phosphorylation. Bars represent the mean ± SEM (n = 8-9) Means with different letters are statistically different from each other ($p<0.05$).

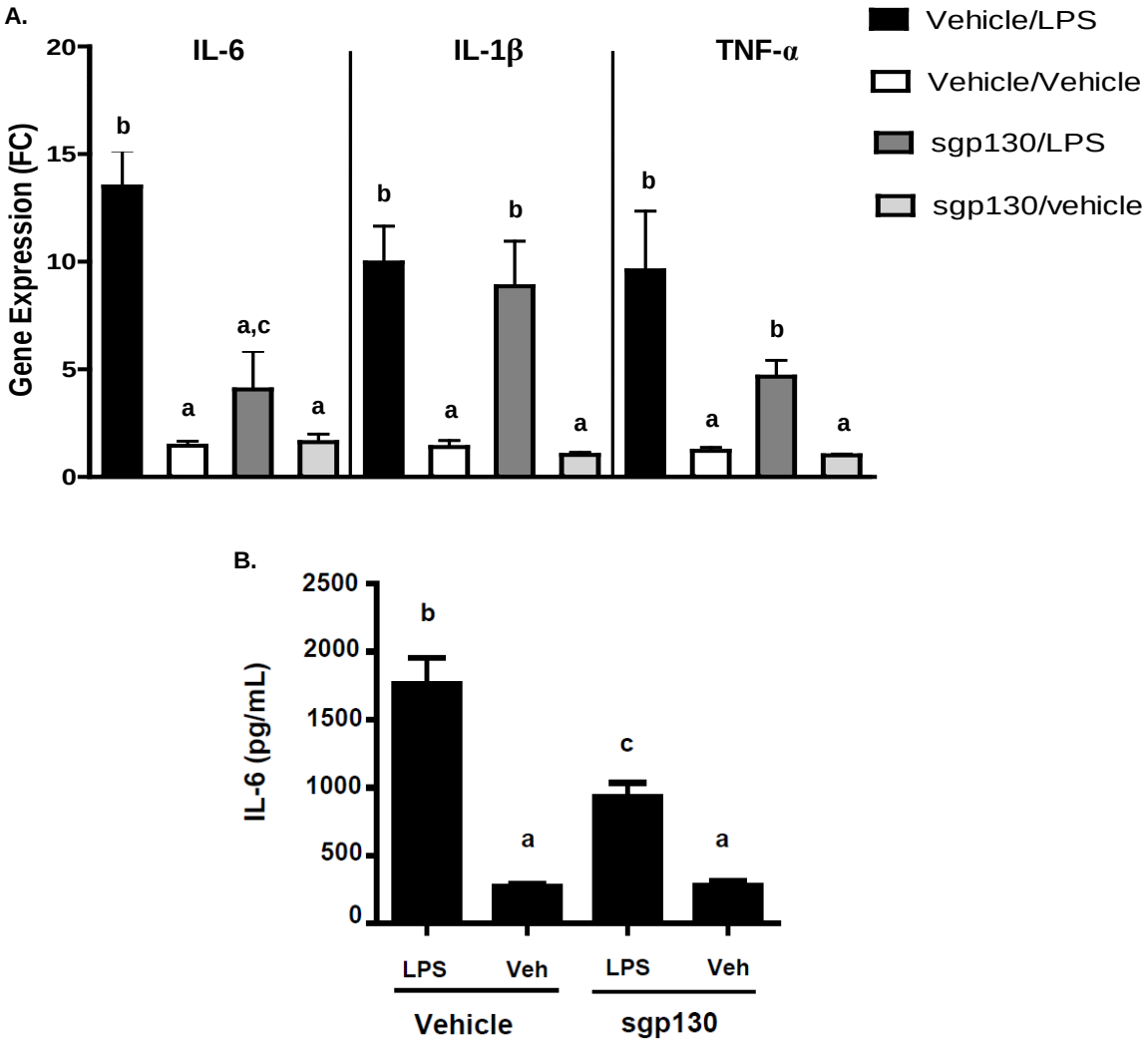


Figure 4.9: sgp130 reduces IL-6 gene expression, and protein secretion *in vivo*. Hippocampal tissue was collected 8 h after icv sgp130 and i.p. LPS and assayed for A) mRNA levels of IL-6¸IL-1β, and TNF-α, B) and IL-6 protein. sgp130 reduced LPS- mRNA levels of IL-6, but not IL-1β or TNF-α, and IL-6 protein in the hippocampus. Bars represent the mean ± SEM (n = 8-9) Means with different letters are statistically different from each other ($p<0.05$).

4.7 Table

Table 1. Plasma cytokines 8 h after sgp130 and LPS.

	Vehicle (icv)		sgp130 (100 ng) (icv)		p-values		
	Saline (i.p.)	**LPS (10 µg) (i.p.)**	**Saline (i.p.)**	**LPS (10µg) (i.p.)**	**sgp130**	**LPS**	**sgp130 × LPS**
IL-1β	206.82 ± 86.19	1113.12 ± 24.48	191.10 ± 85.12	1543 ± 695.96	0.537	0.009*	0.513
IL-6	142.87 ± 117.79	2873.03 ± 994.50	96.50 ± 8.41	2682.06 ± 669.86	0.820	0.001*	0.955
TNF-α	325.89 ± 54.60	987.55 ± 104.77	322.95 ± 73.31	947.93 ± 119.32	0.829	<0.0001*	0.852
IL-10	56.39 ± 23.01	407.34 ± 92.56	46.72 ± 11.47	447.22 ± 174.13	0.764	0.002*	0.719

Bio-Plex Pro™ assay: units = pg/mL
*a significant difference per treatment group ($p<0.05$)

4.8 Literature cited

Abraham, J., Jang, S., Godbout, J.P., Chen, J., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging sensitizes mice to behavioral deficits induced by central HIV-1 gp120. Neurobiology of Aging 29, 614-621.

Abraham, J., Johnson, R.W., 2009a. Central inhibition of interleukin-1 beta ameliorates sickness behavior in aged mice. Brain Behavior and Immunity 23, 396-401.

Abraham, J., Johnson, R.W., 2009b. Consuming a diet supplemented with resveratrol reduced infection-related neuroinflammation and deficits in working memory in aged mice. Rejuvenation Research 12, 445-453.

Atreya, R., Finotto, S., Mudter, J., Mullberg, J., Jostock, T., Holtmann, M., Kishimoto, T., Galle, P.R., Rose-John, S., Neurath, M.F., 2000. Blockade of IL-6 transsignaling abrogates established experimental colitis in mice by suppression of T cell resistance against apoptosis. Gastroenterology 118, 4803.

Berg, B.M., Godbout, J.P., Chen, J., Kelley, K.W., Johnson, R.W., 2005. Alpha-tocopherol and selenium facilitate recovery from lipopolysaccharide-induced sickness in aged mice. Journal of Nutrition 135, 1157-1163.

Berg, B.M., Godbout, J.P., Kelley, K.W., Johnson, R.W., 2004. Alpha-Tocopherol attenuates lipopolysaccharide-induced sickness behavior in mice. Brain Behavior and Immunity 18, 149-157.

Block, M.L., Zecca, L., Hong, J.S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature Reviews: Neuroscience 8, 57-69.

Campbell, I.L., Abraham, C.R., Masliah, E., Kemper, P., Inglis, J.D., Oldstone, M.B.A., Mucke, L., 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin-6. Proceedings of the National Academy of Sciences of the United States of America 90, 10061-10065.

Chen, J., Buchanan, J.B., Sparkman, N.L., Godbout, J.P., Freund, G.G., Johnson, R.W., 2008. Neuroinflammation and disruption in working memory in aged mice after

acute stimulation of the peripheral innate immune system. Brain Behavior and Immunity 22, 301-311.

Chen, Q., Wang, W.C., Bruce, R., Li, H., Schleider, D.M., Mulbury, M.J., Bain, M.D., Wallace, P.K., Baumann, H., Evans, S.S., 2004. Central role of IL-6 receptor signal-transducing chain gp130 in activation of L-selectin adhesion by fever-range thermal stress. Immunity 20, 59-70.

Coles, B., Fielding, C.A., Rose-John, S., Scheller, J., Jones, S.A., O'Donnell, V.B., 2007. Classic interleukin-6 receptor signaling and interleukin-6 trans-signaling differentially control angiotensin II-dependent hypertension, cardiac signal transducer and activator of transcription-3 activation, and vascular hypertrophy in vivo. American Journal of Pathology 171, 315-325.

Combrinck, M.I., Perry, V.H., Cunningham, C., 2002. Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease. Neuroscience 112, 7-11.

Dantzer, R., Bluthe, R.M., Gheusi, G., Cremona, S., Laye, S., Parnet, P., Kelley, K.W., 1998. Molecular basis of sickness behavior. In: Kluger, M.J., Bartfai, T., Dinarello, C.A. (Eds.), Molecular Mechanisms of Fever 132-138.

Diehl, S., Rincon, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. Molecular Immunology 39, 531-536.

Dreuw, A., Radtke, S., Pflanz, S., Lippok, B.E., Heinrich, P.C., Hermanns, H.M., 2004. Characterization of the signaling capacities of the novel gp130-like cytokine receptor. Journal of Biological Chemistry 279, 36112-36120.

Fielding, C.A., McLoughlin, R.M., McLeod, L., Colmont, C.S., Najdovska, M., Grail, D., Ernst, M., Jones, S.A., Topley, N., Jenkins, B.J., 2008. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. Journal of Immunology 181, 2189-2195.

Godbout, J.P., Berg, B.M., Kelley, K.W., Johnson, R.W., 2004. alpha-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6

secretion in primary murine microglia and in brain. Journal of Neuroimmunology 149, 101-109.

Godbout, J.P., Berg, G.M., Krzyszton, C., Johnson, R.W., 2005a. alpha-Tocopherol attenuates NF kappa B activation and pro-inflammatory cytokine production in brain and improves recovery from lipopolysaccharide-induced sickness behavior. Journal of Neuroimmunology 169, 97-105.

Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W., 2005b. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. FASEB Journal 19, 1329.

Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F., Graeve, L., 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochemical Journal 334, 297-314.

Henry, C.J., Huang, Y., Wynne, A., Hanke, M., Himler, J., Bailey, M.T., Sheridan, J.F., Godbout, J.P., 2008. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. Journal of Neuroinflammation 5.

Heyser, C.J., Masliah, E., Samimi, A., Campbell, I.L., Gold, L.H., 1997. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. Proceedings of the National Academy of Sciences of the United States of America 94, 1500-1505.

Huang, Y., Henry, C.J., Dantzer, R., Johnson, R.W., Godbout, J.P., 2008. Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. Neurobiology of Aging 29, 1744-1753.

Jang, S., Dilger, R.N., Johnson, R.W., 2010. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. Journal of Nutrition 140, 140 (110) 1892-1898.

Jang, S., Kelley, K.W., Johnson, R.W., 2008. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proceedings of the National Academy of Sciences of the United States of America 105, 7534-7539.

Jones, S.A., Richards, P.J., Scheller, J., Rose-John, S., 2005. IL-6 transsignaling: The in vivo consequences. Journal of Interferon and Cytokine Research 25, 241-253.

Jones, S.A., Rose-John, S., 2002. The role of soluble receptors in cytokine biology: the agonistic properties of the sIL-6R/IL-6 complex. Biochimica Et Biophysica Acta-Molecular Cell Research 1592, 251-263.

Jostock, T., Mullberg, J., Ozbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M.F., Rose-John, S., 2001. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. European Journal of Biochemistry 268, 160-167.

Kelley, K.W., Bluthe, R.M., Dantzer, R., Zhou, J.H., Shen, W.H., Johnson, R.W., Broussard, S.R., 2003. Cytokine-induced sickness behavior. Brain Behavior and Immunity 17, S112-S118.

Kishimoto, T., Akira, S., Taga, T., 1992. IL-6 receptor and mechanism of signal transduction International Journal of Immunopharmacology 14, 431-438.

Kreutzberg, G.W., 1996. Microglia: A sensor for pathological events in the CNS. Trends in Neurosciences 19, 312-318.

Krzyszton, C.P., Sparkman, N.L., Grant, R.W., Buchanan, J.B., Broussard, S.R., Woods, J., Johnson, R.W., 2008. Exacerbated fatigue and motor deficits in interleukin-10-deficient mice after peripheral immune stimulation. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 295, R1109-R1114.

Laflamme, N., Rivest, S., 2001. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. FASEB Journal 15, 155-163.

Maier, S.F., Goehler, L.E., Fleshner, M., Watkins, L.R., 1998. The role of the vagus nerve in cytokine-to-brain communication. Neuroimmunomodulation 840, 289-300.

Maier, S.F., Watkins, L.R., 1998. Cytokines for psychologists: Implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. Psychological Review 105, 83-107.

Marz, P., Otten, U., Rose-John, S., 1999. Neural activities of IL-6-type cytokines often depend on soluble cytokine receptors. European Journal of Neuroscience 11, 2995-3004.

McLoughlin, R.M., Jenkins, B.J., Grail, D., Williams, A.S., Fielding, C.A., Parker, C.R., Ernst, M., Topley, N., Jones, S.A., 2005. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proceedings of the National Academy of Sciences of the United States of America 102, 9589-9594.

Mines, M.A., Yuskaitis, C.J., King, M.K., Beurel, E., Jope, R.S., 2010. GSK3 influences social preference and anxiety-related behaviors during social Interaction in a mouse model of fragile X syndrome and autism. PloS One 5.

Mizuguchi, H., Mizuno, H., Yasukawa, K., Ishiguro, T., Fukui, K., Imanaka, T., Takagi, M., 2001. Enhanced signal transduction by a directly fused protein of interleukin-6 and its receptor. Journal of Bioscience and Bioengineering 91, 299-304.

Modrell, B., Liu, J.W., Miller, H., Shoyab, M., 1994. LIF and OSM directly interact with a soluble form of gp130, the IL-6 receptor signal-transducing subunit. Growth Factors 11, 81-91.

Mudter, J., Neurath, M.F., 2007. IL-6 signaling in inflammatory bowel disease: Pathophysiological role and clinical relevance. Inflammatory Bowel Diseases 13, 1016-1023.

Nancey, S., Hamzaoui, N., Moussata, D., Graber, I., Bienvenu, J., Flourie, B., 2008. Serum interleukin-6, soluble interleukin-6 receptor and Crohn's disease activity. Digestive Diseases and Sciences 53, 242-247.

Quan, N., 2008. Immune-to-brain signaling: How important are the blood-brain barrier-independent pathways? Molecular Neurobiology 37, 142-152.

Ransohoff, R.M., Perry, V.H., 2009. Microglial physiology: Unique stimuli, specialized responses. Annual Review of Immunology 27, 119-145.

Richards, P.J., Nowell, M.A., Horiuchi, S., McLoughlin, R.M., Fielding, C.A., Grau, S., Yamamoto, N., Ehrmann, M., Rose-John, S., Williams, A.S., Topley, N., Jones, S.A., 2006. Functional characterization of a soluble gp130 isoform and its therapeutic

capacity in an experimental model of inflammatory arthritis. Arthritis and Rheumatism 54, 1662-1672.

Richwine, A.F., Godbout, J.P., Berg, B.M., Chen, J., Escobar, J., Millard, D.K., Johnson, R.W., 2005. Improved psychomotor performance in aged mice fed diet high in antioxidants is associated with reduced ex vivo brain interleukin-6 production. Brain Behavior and Immunity 19, 512-520.

Rosejohn, S., Heinrich, P.C., 1994. SOLUBLE RECEPTORS FOR CYTOKINES AND GROWTH-FACTORS - GENERATION AND BIOLOGICAL FUNCTION. Biochemical Journal 300, 281-290.

Schobitz, B., Pezeshki, G., Pohl, T., Hemmann, U., Heinrich, P.C., Holsboer, F., Reul, J., 1995. Soluble interleukin-6 (IL-6) receptor augments central effects of IL-6 in-vivo. FASEB Journal 9, 659-664.

Sparkman, N.L., Buchanan, J.B., Heyen, J.R.R., Chen, J., Beverly, J.L., Johnson, R.W., 2006. Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. Journal of Neuroscience 26, 10709-10716.

Sparkman, N.L., Johnson, R.W., 2008. Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. Neuroimmunomodulation 15, 323-330.

Swiergiel, A.H., Dunn, A.J., 1999. The roles of IL-1, IL-6, and TNF alpha in the feeding responses to endotoxin and influenza virus infection in mice. Brain Behavior and Immunity 13, 252-265.

Taga, T., Kishimoto, T., 1997. gp130 and the interleukin-6 family of cytokines. Annual Review of Immunology 15, 797-819.

Ye, S.M., Johnson, R.W., 1999. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Zorina, Y., Iyengar, R., Bromberg, K.D., 2010. Cannabinoid 1 receptor and interleukin-6 receptor together induce integration of protein kinase and transcription factor signaling to trigger neurite outgrowth. Journal of Biological Chemistry 285, 1358-1370.

Chapter 5

Interleukin-6 *trans*-signaling in the senescent mouse brain is involved in infection-related deficits in contextual fear conditioning

5.1 Abstract

Excessive production of pro-inflammatory cytokines in the senescent brain in response to peripheral immune stimulation is thought to induce behavioral pathology, however, few studies have examined if the increase in pro-inflammatory cytokines is accompanied by an increase in cytokine signaling. Here, we focused on IL-6 as a prototypic pro-inflammatory cytokine and used phosphorylated STAT3 as a marker of IL-6 signaling. In an initial study, IL-6 mRNA and the magnitude and duration of STAT3 activation were increased in the hippocampus of senescent mice compared to adults after i.p. injection of lipopolysaccharide (LPS). The LPS-induced increase in STAT3 activity was ablated in aged IL-$6^{-/-}$ mice, suggesting IL-6 is a key driver of STAT3 activity in the aged brain. To determine if IL-6 activated the classical or *trans*-signaling pathway, before receiving LPS i.p., aged mice were injected icv with sgp130, an antagonist of the *trans*-signaling pathway. Importantly, the LPS-induced increases in both IL-6 and STAT3 activity in the hippocampus were inhibited by sgp130. To assess hippocampal function, aged mice were injected icv with sgp130 and i.p. with LPS immediately after the acquisition phase of contextual fear conditioning, and immobility was assessed in the retention phase 48 hours (h) later. LPS reduced immobility in aged mice, indicating immune activation interfered with memory consolidation. However, sgp130 blocked the deficits in contextual fear conditioning caused by LPS. Taken together, the results suggest IL-6 *trans*-signaling is increased in the senescent brain following peripheral

LPS challenge and that sgp130 may protect against infection-related neuroinflammation and cognitive dysfunction in the aged.

5.2 Introduction

Interleukin-6 (IL-6) has a role in cognitive dysfunction. For example, immunoneutralization of IL-6 lengthened long-term potentiation (LTP) and improved spatial alternation behavior (Balschun et al., 2004) while IL-6-deficient mice were refractory to lipopolysaccharide (LPS)-induced deficits in working memory (Sparkman et al., 2006). IL-6 also disrupts neurogenesis (Vallieres et al., 2002) and is up regulated in an array of neurodegenerative diseases (Gruol and Nelson, 1997; Hofmann et al., 2009; Licastro et al., 2003; Muller et al., 1998). Furthermore, in senescence, constitutive expression of IL-6 by brain microglia is up regulated (Godbout and Johnson, 2004; Ye and Johnson, 1999, 2001) and during infection there is excessive production of pro-inflammatory cytokines including IL-6, leading to prolonged sickness behavior (Abraham and Johnson, 2009; Godbout et al., 2005; Huang et al., 2008), cognitive deficits (Barrientos et al., 2006; Chen et al., 2008; Rosczyk et al., 2008), affective disorders such as increased anxiety and depression (Godbout et al., 2008; Kiecolt-Glaser et al., 2003), and even atrophy of neurons (Richwine et al., 2008). These findings in rodents have led us to speculate that the acute cognitive disorders seen in older adults with an infection are due to excessive production of pro-inflammatory cytokines in the brain, although very little is known regarding the effects of aging on cytokine receptor signaling.

The IL-6 receptor is activated through two separate, but related pathways termed classical and *trans*-signaling. The receptor consists of two subunits: the IL-6 receptor-

alpha chain (IL-6R), which binds IL-6, and the transmembrane signaling subunit, glycoprotein 130 (gp130), which is the intracellular signal transducer and is expressed across all cell types. Classical activation consists of the IL-6 ligand binding to the membrane-bound IL-6R. It is important to note that both receptor subunits (IL-6R and gp130) can be cleaved immediately before the membrane spanning region by alternative splicing or shed by proteolytic enzymes to produce a soluble receptor located in extracellular matrix. The expression of membrane-bound IL-6R is generally limited to a few cells of the immune system, while gp130 is ubiquitously expressed (Heinrich et al., 1998; Kishimoto et al., 1992). Thus, the basis of *trans*-signaling is the ability of soluble IL-6R (sIL-6R) to bind IL-6 in the extracellular compartment to form an IL-6/sIL-6R complex; this complex has an increased binding affinity to membrane-bound gp130 when compared to IL-6 alone. This mechanism of action confers IL-6 responsiveness in any cell type that expresses gp130 (Jones et al., 2005; Rose-John and Heinrich, 1994).

Upon binding through either the classical or *trans*-signaling pathway, gp130 dimerizes and autophosphorylates, resulting in the activation of Janus kinase-1 and 2 (Jak1 and Jak2). These tyrosine kinases phosphorylate the cytoplasmic region of gp130 creating recruitment sites for signal transducer and activation of transcription-3 (STAT3), a Src-homology-2 (SH2) domain-containing signaling molecule. Activated STAT3 forms a dimer, autophosphorylates, and translocates to the nucleus where it binds to enhancer elements of the IL-6 promoter region. Thus the main consequence of both classical or *trans*-signal IL-6 receptor action is to induce gene transcription and subsequent synthesis and secretion of IL-6, although *trans*-signaling allows this in many

more cell types (Heinrich et al., 1998). sIL-6R and soluble gp130 (sgp130) have varying effects on circulating IL-6. sgp130 acts as a partial antagonist, or decoy receptor, by binding the IL-6/sIL-6R complex and preventing it from binding membrane-bound gp130 to initiate signal transduction (Jostock et al., 2001).

Neurons do not seem to express appreciable amounts of IL-6R, however, they do express large amounts of gp130 (Burton et al., 2011; Schobitz et al., 1993), alluding to the importance of IL-6 *trans*-signaling in the brain. Indeed IL-6 *trans*-signaling in the brain was recently shown to play a pivotal role in mediating LPS-induced sickness behavior (Burton et al., 2011). Other studies have begun to reveal the anti- versus pro-inflammatory actions of IL-6, as *trans*-signaling appears to elicit a pro-inflammatory response by causing non-immune cells to produce IL-6 which in turn recruits immune cells to the local site (Barkhausen et al., 2011; Barrientos, 2011; Greenhill et al., 2011; Scheller et al., 2011).

Although IL-6 is increased in the brain of senescent mice, to our knowledge there have been no studies examining the extent to which peripheral infection influences IL-6 *trans*-signaling in the brain or its role in cognitive dysfunction. Thus, the present study investigated hippocampal STAT3 activation as a marker for IL-6 signaling and hippocampal-dependent learning and memory in aged mice after peripheral injection of LPS. We further investigated the extent to which inhibition of IL-6 *trans*-signaling with sgp130 would mitigate STAT3 activation and deficits in learning and memory. The important results suggest that IL-6 *trans*-signaling is increased in the hippocampus of aged mice after LPS and that IL-6 *trans*-signaling plays a key role in LPS-induced deficits in hippocampal-dependent learning and memory.

5.3 Materials and Methods

Animals and surgery

Adult (3-6 months) and aged (22-24 months) male BALB/c, C57BL/6 (IL-$6^{+/+}$) and IL-6 knockout B6.129S2-Il6tm1 Kopf/J (IL-$6^{-/-}$) (Kopf et al., 1994) mice were used. All BALB/c mice were obtained from our in-house colony whereas the IL-$6^{+/+}$ and IL-$6^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). The IL-$6^{+/+}$ and IL-$6^{-/-}$ mice were 2-months old upon receipt. Mice were housed in polypropylene cages and maintained at 21°C under a reverse-phase 12-h light-dark cycle with *ad libitum* access to water and rodent chow. At the end of each study, mice were examined post mortem for gross signs of disease (e.g., tumors or splenomegaly). Data from mice determined to be unhealthy were excluded from the analysis (< 5%).

Surgery: For some experiments intracerebroventricular (icv) cannulation was performed as described previously (Abraham et al., 2008). Immediately after surgery and again 8-12 hours (h) later mice received buprenorphine (0.05 mg/kg s.c.) to aid with any post-operative discomfort. Mice were provided a minimum of 7 days to recover before initiating an experiment. Accurate placement of the cannula was confirmed by allowing 2 µl of sterile saline to flow via gravity into the lateral ventricle. When cannula placement could not be confirmed, mice were excluded from the study. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Experimental protocols

Mice were handled 1-2 minutes (min) each day for 7 days before experimentation to acclimate them to handling. To assess the effects of LPS on STAT3 phosphorylation in the hippocampus as well as IL-6 in the hippocampus mice were injected i.p. with sterile saline or 0.33 mg/kg BW (10 µg) LPS (serotype 0127:B8, obtained from Sigma, St. Louis, MO) and killed by CO_2 asphyxiation 1, 2, 4, 6, or 8 h later. Blood samples were collected via cardiac puncture into EDTA-coated syringes to obtain plasma, and the brain was rapidly removed and dissected to obtain hippocampal tissue. Plasma and hippocampal tissue were snap frozen in liquid nitrogen and stored at -80^{o} C for later analysis. To assess the role of IL-6 *trans*-signaling mice were injected icv with sterile saline containing 0.1% BSA (vehicle) or 100 ng sgp130 (R&D systems, Minneapolis, MN) in 2 µl vehicle and then i.p. with sterile saline or 0.33 mg/kg BW (10 µg) LPS. In some cases mice were killed to obtain hippocampal tissue as described above. In other cases, mice were evaluated in a contextual fear conditioning paradigm to assess hippocampal-dependent learning and memory.

Western immunoblotting

To assess IL-6 signaling, hippocampal tissue was unthawed, and lysed in ice cold lysis buffer containing: 100 mM HEPES (7.5 pH), 150 mM NaCl, 1% nonidet P-40 (U.S. Biological, Swampscott, MA), 2 mM EGTA, 2 mM sodium orthovanadate, protease inhibitor cocktail (10 0mM EDTA, 1 µg/mL AEBSF, bestatin, pepstatin A, leupeptin, aprotinin, and E-64), and 1 mM PMSF, and centrifuged at 11,000 × g for 10 min at 4^{o}C to remove all cellular debris. Protein concentration was determined using the BCA Protein Assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA). Lysate

concentration was then normalized and denatured in SDS/PAGE buffer at 95^{o}C and stored at -20^{o}C until use. All lysates were electrophoresed and separated on a 7.5% SDS-PAGE gel, and transferred onto nitrocellulose membranes (GE Healthcare, Minneapolis, MN). The membranes were blocked with 5% non-fat milk and incubated with anti-phosphorylated STAT3 (tyr-705) antibody (Cell Signaling, Danvers, MA) overnight at 4°C. After incubation with an HRP-conjugated secondary antibody, the protein bands were detected with a chemiluminescent substrate (Cell Signaling, Danvers, MA) and Bio-Max film (Eastman Kodak Company, Rochester, NY). For detection of total STAT3 protein, the membranes were stripped with stripping buffer (2% SDS, 6.25 mM Tris.HCL [6.8 pH], 0.704% β-ME), followed by overnight incubation with anti-STAT3 antibody (Cell Signaling, Danvers, MA) at 4°C. The phosphorylated STAT3 protein has an alpha and beta isoform which are expressed in two bands at 86 and 79kDa, respectively. Upon activation, the alpha isoform (86-kDa) is the principal isoform that is phosphorylated and the beta isoform (79-kDa) represents a point of maximal STAT3 activation. The area where both bands are located were quantified using ImageJ 1.41 software (NIH).

Interleukin-6 mRNA measurement by quantitative real-time PCR

Total RNA from hippocampal tissue was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO). A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's protocol. Quantitative real-time PCR was performed using the Applied Biosystems (Foster, CA) Assay-on Demand Gene Expression protocol as previously described (Krzyszton et al., 2008). In brief, cDNA was amplified

by PCR where a target cDNA (IL-6, Mm00446190_m1; and a reference cDNA (glucose-3 phosphate dehydrogenase, Mm99999915_g1) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR reactions were performed in triplicate under the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec, and 60^{o}C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, and results are expressed as fold difference.

Interleukin-6 detection in hippocampus and plasma

Hippocampal tissue was lysed in ice cold lysis buffer and protein concentrations were determined using the BCA protein assay according to manufacturer's protocol. The antibodies and standards for the IL-6 ELISA were used according to the description by the manufacturer (eBiosciences San Diego, CA). Plasma samples were assayed for IL-6 using a bead-based immunoassay kit combined with a Cytokine Reagent kit, as described by the manufacturer (Bio-Rad, Hercules, CA).

Contextual fear conditioning

A contextual fear conditioning paradigm was used as described previously with few modifications (Peleg et al., 2010). The fear conditioning apparatus consisted of an opaque conditioning cage (30 × 30 × 30 cm) with a transparent ceiling to permit video recording by an overhead camera. The floor consisted of a series of stainless steel rods wired to a shock generator and scrambler (ENV-414S, MED Associates, St. Albans, VT). On acquisition day, mice were placed in the cage for 120 s, followed by a

2-s foot-shock (0.75 mA); this time interval was repeated and 30 s after the delivery of the second shock mice were co-administered saline or sgp130 icv and saline or LPS i.p. To test the hippocampal-dependent contextual fear conditioning, 48 h after acquisition mice were placed in the original conditioning cage for the time equal to that in acquisition (4 min and 30 s). A trained observer who was blind to experimental treatments scored the fearful experience by a continuous measurement of freezing (complete immobility), the dominant fear behavioral response (Fanselow, 2000). In interpreting data from the fear conditioning paradigm, an increase in freezing in the retention phase (conducted 48 h after acquisition) indicates an improvement in learning.

Statistical analysis

All data were analyzed using the ANOVA routine in Statview and MIXED procedure of the Statistical Analysis System software (SAS Inst., Cary, NC). Data were subjected to a univariate analysis to ensure normality. In the time course and knockout study, STAT3 phosphorylation, IL-6 mRNA and protein were subjected to a two-way ANOVA in which age (adult or aged) and LPS (sterile saline or 10 µg) were between subject measures. Percent freezing on retention day, IL-6 protein levels, and STAT3 phosphorylation were subjected to a two-way ANOVA in which sgp130 (vehicle or 100 ng), and LPS (sterile saline or 10 µg) were between subject measures. When appropriate, data from individual time points were subjected to ANOVA to determine treatment effects. Post hoc Student's *t* test of least square means was used to determine if treatment means were significantly different from one another ($p<0.05$). All data are presented as mean ± standard error of the mean (SEM).

5.4 Results

LPS-induced STAT3 phosphorylation is increased in hippocampus of aged mice

As a read out for IL-6 signaling, STAT3 phosphorylation was determined in the hippocampus of adult and aged mice after i.p. injection of LPS. Figure 5.1 shows representative western blots revealing phosphorylated STAT3 in hippocampi collected from adult and aged mice at various times after injection of LPS and the mean STAT3 phosphorylation from 8 or 9 treatment replicates at each time point for each age. As expected STAT3 activity in the hippocampus was increased 1 h after LPS in both adult and aged animals. However, whereas the level of STAT3 phosphorylation reached a plateau by 1 h in adults, it continued to increase in the aged. An age × LPS interaction at 4 h ($p<0.05$) and at 6 h ($p<0.01$) indicated a marked increase in STAT3 activity in the hippocampus of aged mice. As we surmised that increased IL-6 was responsible for the increased STAT3 activity, plasma IL-6 and IL-6 mRNA in the hippocampus were measured in adult and aged mice 6 h after LPS injection when STAT3 phosphorylation was maximal in the aged. As evident in Figure 5.2, before or after LPS injection, plasma IL-6 (Figure 5.2A) and hippocampal IL-6 mRNA (Figure 5.2B) were markedly higher in aged mice compared to adults. Collectively, these data show a close association between increased levels of IL-6 and IL-6 signaling during times of peak sickness in the hippocampus of senescent mice. To confirm that IL-6 was responsible for the increased STAT3 activity, a subsequent study was conducted using adult and aged IL-$6^{+/+}$ and IL-$6^{-/-}$ mice. Figure 5.3 shows representative western blots revealing phosphorylated STAT3 in hippocampi collected from adult and aged IL-$6^{+/+}$ and IL-$6^{-/-}$ mice 6 h after injection of LPS and the mean STAT3 phosphorylation from 6 or 7 treatment replicates.

Despite employing a different genetic background than in the first study (C57BL/6 vs. BALB/c), once again hippocampal STAT3 phosphorylation was significantly increased in aged IL-$6^{+/+}$ mice compared to adult IL-$6^{+/+}$ mice ($p<0.05$) after LPS injection (Figure 5.3B). Importantly, the LPS-induced increase in STAT3 activity was ablated in IL-$6^{-/-}$ mice (Figure 5.3B), suggesting IL-6 is a key driver of STAT3 activity in the aged brain after peripheral immune stimulation.

IL-6 *trans*-signaling induces STAT3 activity in hippocampus of aged mice

To determine if the age-related increase in hippocampal STAT3 activity after LPS injection was mediated by IL-6 via the classical or *trans*-signaling pathway, aged BALB/c mice were surgically fit with an indwelling icv cannula. Following recovery, mice were injected icv with sgp130 and i.p. with LPS. Similar to earlier results, LPS up regulated STAT3 phospho-protein and IL-6 in the hippocampus (Figures 5.4 and 5.5, respectively). Furthermore, there was a sgp130 × LPS interaction where both LPS-induced IL-6 ($p<0.04$) and STAT3 activity ($p<0.05$) in the hippocampus, was markedly reduced by sgp130. LPS is a potent activator of IL-6 and the positive feedback loop which induces optimal expression of STAT3 in the vehicle/LPS treated group, but this expression is ameliorated in sgp130/LPS treated animals. As sgp130 binds IL-6 that is coupled with sIL-6R preventing it from activating membrane bound gp130 (Barkhausen et al., 2011), these data suggest a key role for IL-6 *trans*-signaling in the hippocampus.

sgp130 inhibits LPS-induced deficits in contextual fear conditioning

Circulating IL-6 and cognitive dysfunction are positively correlated (Bellinger et al., 1995; Gruol and Nelson, 1997; Maggio et al., 2006; Weaver et al., 2002), and acute cognitive disorders are common in elderly patients during peripheral infection. Thus, we

were eager to know if inhibiting IL-6 *trans*-signaling would protect aged mice from LPS-induced deficits in cognition. Aged BALB/c mice were injected icv with sgp130 and i.p. with LPS immediately after the acquisition phase of contextual fear conditioning. Forty-eight hours later their freezing response in the retention phase was assessed. As evident in Figure 5.6, LPS reduced immobility in aged mice, indicating immune activation interfered with memory consolidation. However, icv injection of sgp130 which reduced STAT3 activation and IL-6 in the hippocampus completely blocked the deficit in hippocampal-based contextual fear conditioning caused by LPS.

5.5 Discussion

Excessive production of pro-inflammatory cytokines in the senescent brain in response to peripheral immune stimulation (Godbout and Johnson, 2009) is thought to induce behavioral pathology and be a forerunner to neurodegenerative disease. However, few studies have examined if the increase in pro-inflammatory cytokines is accompanied by an increase in cytokine signaling, which would be a prerequisite if the behavioral changes are driven by the pro-inflammatory cytokine response. By focusing on IL-6 as a prototypical pro-inflammatory cytokine, the present results show that the magnitude and duration of IL-6 signaling are increased in the hippocampus of senescent mice compared to adults after peripheral injection of LPS. They further suggest a key role for IL-6 *trans*-signaling in hippocampal dysfunction in the senescent and highlight the potential value of sgp130 for preventing infection-related behavioral pathology.

In the present study, STAT3 phosphorylation was used as an indicator of IL-6 signaling. Both classical and *trans*-signaling pathways cause gp130 to dimerize,

resulting in an intracellular cascade that recruits and phosphorylates STAT3. Although other cytokines that were previously detected in the CNS also activate STAT3 (e.g., IL-10), the fact that STAT3 activation was not increased after LPS injection in aged IL-$6^{-/-}$ mice strongly suggests the increased STAT3 phosphorylation in aged wild type mice was due to IL-6. This is further supported by the fact that IL-6 mRNA and protein were markedly higher in the hippocampus of aged mice compared to adults at the time corresponding to maximal STAT3 phosphorylation. However, it should be noted that other cytokines that can activate STAT3 were not measured in this study. Due to the fact that sgp130 binds the IL-6-sIL-6R complex in the extracellular matrix and prevents it from activating membrane bound gp130, it is a naturally occurring antagonist of the IL-6 *trans*-signaling pathway (Jostock et al., 2001). Since it has a low affinity for IL-6 that is unbound to sIL-6R, sgp130 does not interfere with IL-6R signaling (Jones et al., 2005; Muller-Newen et al., 1998). Therefore, sgp130 can be used to elucidate the contribution of the IL-6 *trans*-signaling pathway to biologically relevant outcomes. For example, sgp130 was recently reported to reduce LPS-induced sickness behavior in mice (Burton et al., 2011). The finding that LPS-induced sickness was mediated by the *trans*-signaling pathway and not the classical pathway was consistent with the finding that Neuro.2A cells (a neuronal cell line) expressed abundant levels of gp130 but very little IL-6R (Burton et al., 2011). If neurons express few IL-6R but are laden with gp130 and mostly influenced by IL-6 through the *trans*-signaling pathway, this would explain earlier work that found recombinant IL-6 had little effect on behavior when injected icv but induced profound sickness when co-administered with sIL-6R (Schobitz et al., 1995). In the present study sgp130 given icv reduced LPS-induced STAT3 activity in the

hippocampus of aged mice, further highlighting the importance of IL-6 *trans*-signaling in the brain. These findings regarding IL-6 *trans*-signaling in the brain are consistent with a recent study of IL-6 *trans*-signaling and TLR4-driven inflammatory responses in the periphery (Greenhill et al., 2011). Cross-talk between JAK/STAT and TLR4 pathways is now viewed as a broad-based mechanism that regulates the severity of inflammation. Other studies suggest inhibiting IL-6 *trans*-signaling with sgp130 may be helpful in treating a number of inflammatory conditions including arthritis, peritonitis, and colitis (Coles et al., 2007; McLoughlin et al., 2005; Richards et al., 2006).

Activated STAT3 translocates to the nucleus where it binds to enhancer elements of the IL-6 gene to induce transcription. Thus, a primary effect of IL-6 is to induce expression of more IL-6 (Aaronson and Horvath, 2002). Other IL-6 family members (LIF, OSM, IL-11) as well as IL-10 and interferons activate STAT3, albeit, to a lesser degree, and evidence from this (Figure 3) and other studies, show that in IL-6 is the most potent activator of STAT3 (Bluthe et al., 2000). Although our goal was to investigate IL-6 signaling in the senescent brain as opposed to mechanisms responsible for the exaggerated production of the cytokine, the present study suggests the involvement of a positive feedback loop initiated through *trans*-signaling where IL-6 begets IL-6. This is best exemplified in Figure 5.5 where sgp130 given icv is shown to reduce LPS-induced IL-6 in the hippocampus by about half.

Due to the fact that sgp130 reduced STAT3 activity and IL-6 levels in the hippocampus, it was reasonable to postulate that it would also inhibit LPS-induced deficits in hippocampal-dependent learning and memory. Therefore, we chose to focus on hippocampal-dependent context conditioning since it has been shown that the

hippocampus is greatly affected in aging. This is important because immune stimuli are known to inhibit consolidation of memories in hippocampal-dependent tasks (Pugh, 2001; Pugh et al., 1998), and hippocampal-dependent tasks are more easily disrupted in aged animals compared to adults (Adams et al., 2001; Barrientos et al., 2006; Bruunsgaard et al., 2001; Chen et al., 2008; Cortese et al., 2011) In the present study, aged mice given LPS after the acquisition phase showed less immobility when reintroduced to the environment where they experienced mild shock, meaning they were less effective at establishing an association between the environment and noxious stimulus, which could be dangerous in a natural setting. However, treatment with sgp130 completely blocked this effect of LPS, suggesting IL-6 *trans*-signaling is involved in infection-related hippocampal dysfunction. Consistent with previous studies, results with adult animals showed context conditioning was impaired by post-training LPS, although the age × treatment interaction did not reach significance (data not shown). This phenomenon is potentially due to the single point in time of testing, which was not broad enough to reveal differences.

Ongoing studies are underway to identify the cell type(s) in the brain that are responsible, and the mechanisms behind this IL-6 *trans*-signaling -mediated response to LPS during aging; proposed mechanisms are modulation of the blood-brain barrier, chemokine activation, and oxidative stress. It is interesting that blocking *trans*-signaling was sufficient to protect contextual fear conditioning since other pro-inflammatory cytokines such as IL-1β are known to inhibit hippocampal-dependent learning and memory during infection (Yirmiya et al., 2002), but signal through different pathways. However, we previously showed that mice deficient in IL-6 (i.e., IL-$6^{-/-}$ mice) also had

reduced levels of IL-1β and TNFα in the hippocampus after peripheral LPS challenge, suggesting IL-6 plays a permissive role in the production and/or activities of other pro-inflammatory cytokines (Sparkman et al., 2006). This is consistent with a recent study by Greenhill et al. (2011) that suggested IL-6 *trans*-signaling is an important determinant in TLR4-driven inflammation. Taken together, the present study suggests that sgp130 may be useful for protecting against infection-related neuroinflammation and cognitive dysfunction in the aged.

5.6 Figures

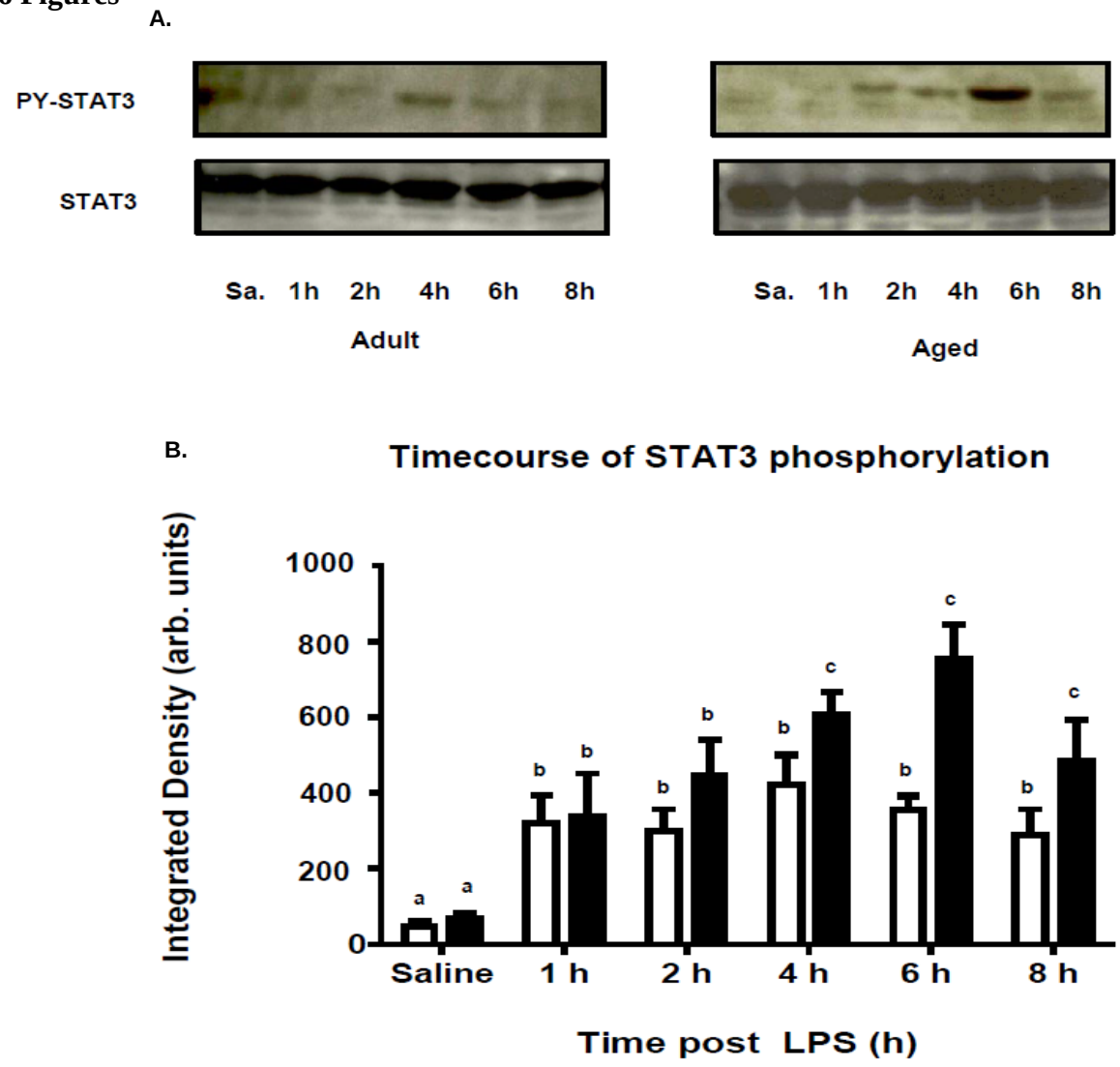


Figure 5.1: Activated STAT3 in the hippocampus of adult and aged mice after peripheral injection of LPS. Adult and aged BALB/c mice were injected i.p. with LPS and hippocampal tissue was collected at various time points after injection to measure phosphorylated STAT3. The upper panel shows representative western blots and the bar graph shows mean STAT3 phosphorylation (± SEM) from 8-9 treatment replicates at each time point for each age (because STAT3 activity was not affected in saline controls over time, data from saline treated mice at different time points was pooled. Means with different letters are significantly different ($p<0.05$).

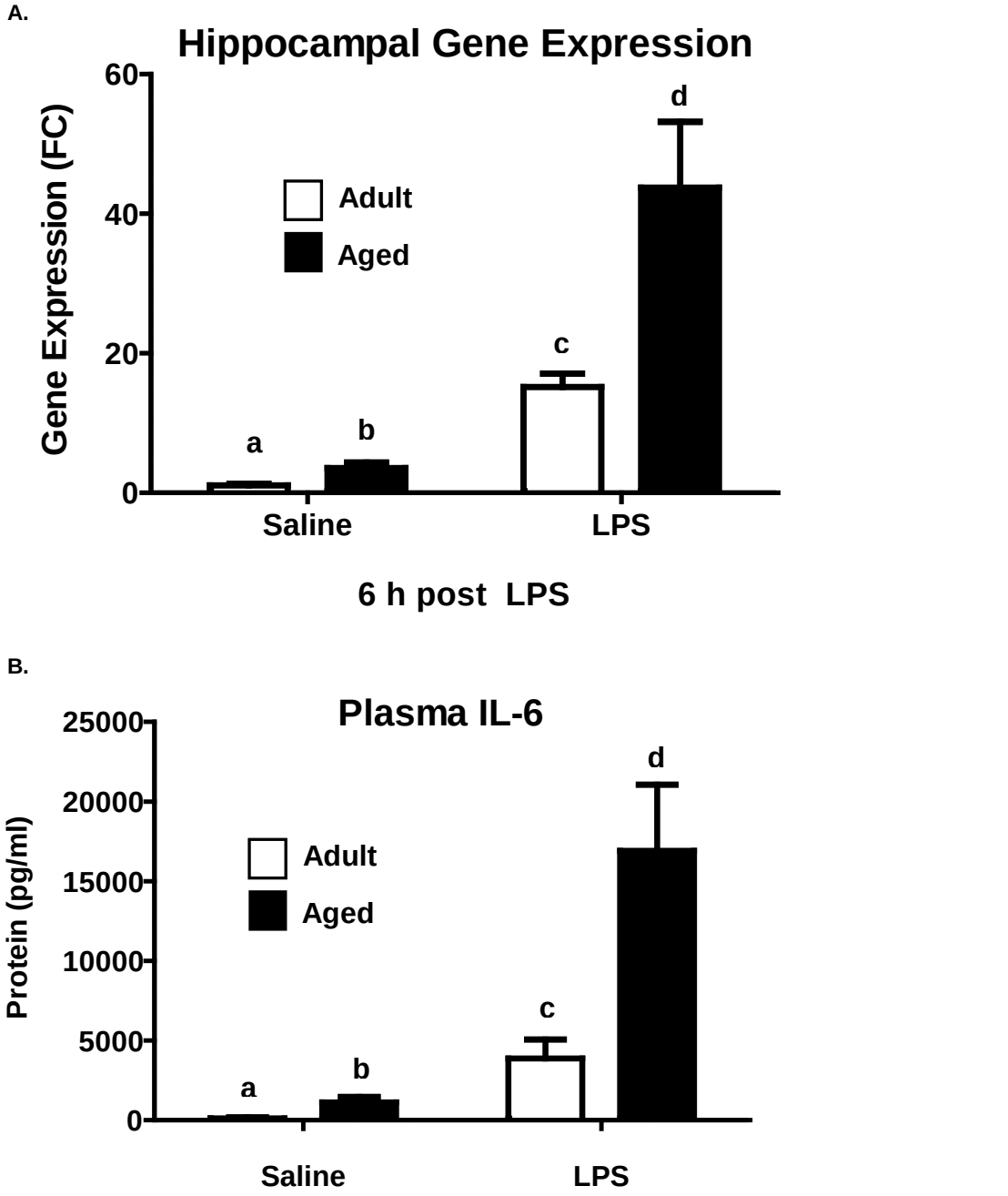


Figure 5.2: Plasma IL-6 and IL-6 mRNA in the hippocampus in aged and adult mice 6 h after LPS injection. Adult and aged BALB/c mice were injected i.p. with LPS and 6 h later, blood and brain tissue were collected for analysis. Bars represent the mean ± SEM (n = 7-8). Means with different letters are significantly different ($p<0.05$).

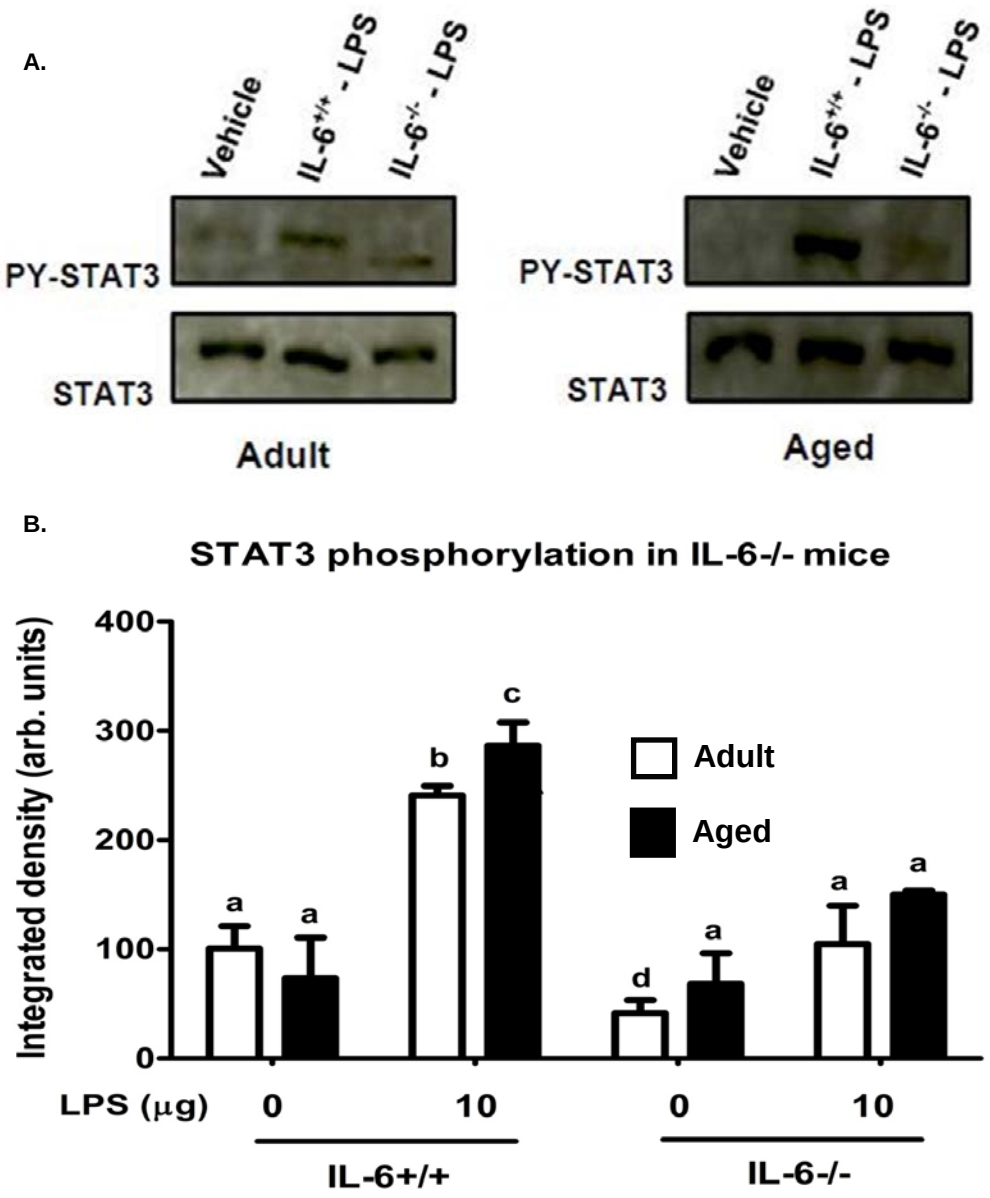


Figure 5.3: Phosphorylated STAT3 in hippocampus of adult and aged IL6$^{+/+}$ and IL-6$^{-/-}$ mice after injection of LPS. Adult and aged IL-6$^{+/+}$ and IL-6$^{-/-}$ mice were injected i.p. with LPS and hippocampal tissue was collected to measure phosphorylated STAT3. The upper panel shows representative western blots and the bar graph shows mean STAT3 phosphorylation (± SEM) from 6-7 treatment replicates. Means with different letters are significantly different ($p<0.05$).

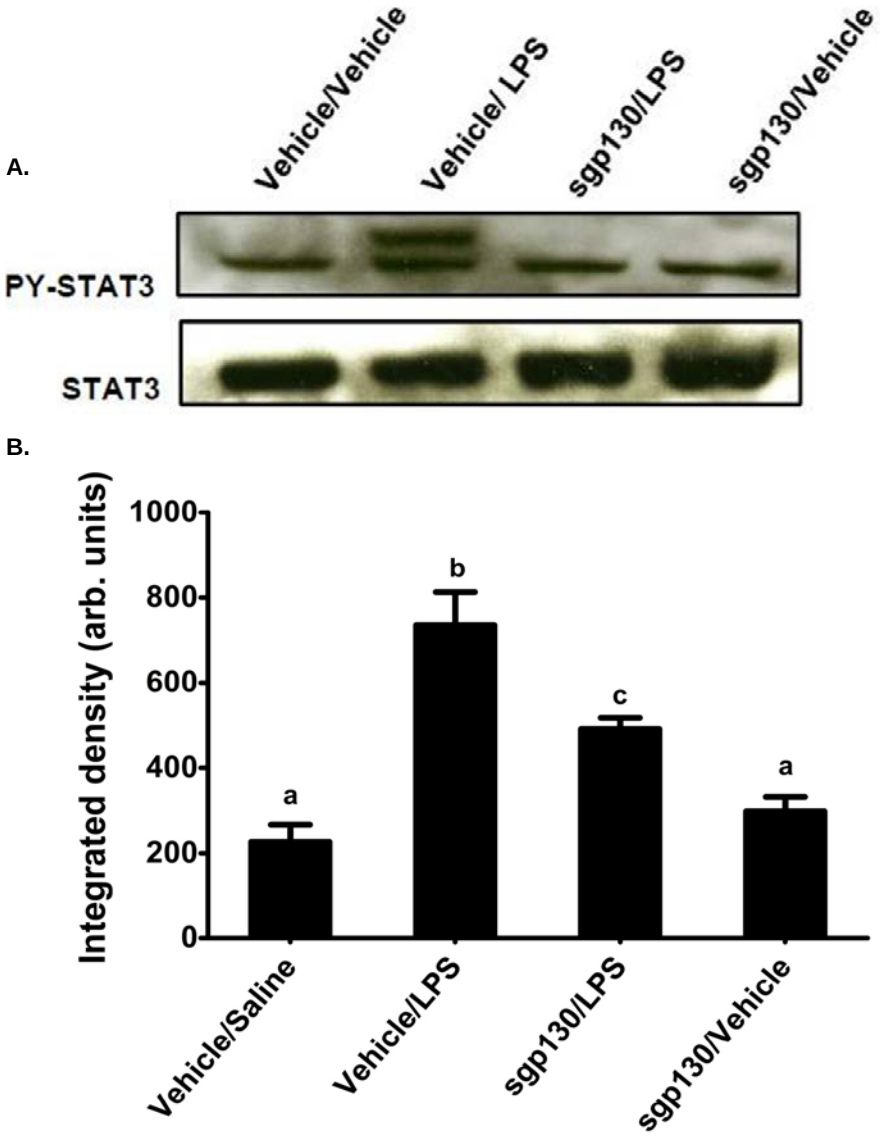


Figure 5.4: Effects of sgp130 on LPS-induced STAT3 activity in the hippocampus of aged mice. Aged BALB/c mice were injected icv with sgp130 and i.p. with LPS and hippocampal tissue was collected 6 h later. The upper panel shows representative western blots and the bar graph shows mean STAT3 phosphorylation (± SEM) from 8-9 treatment replicates. Means with different letters are significantly different ($p<0.05$).

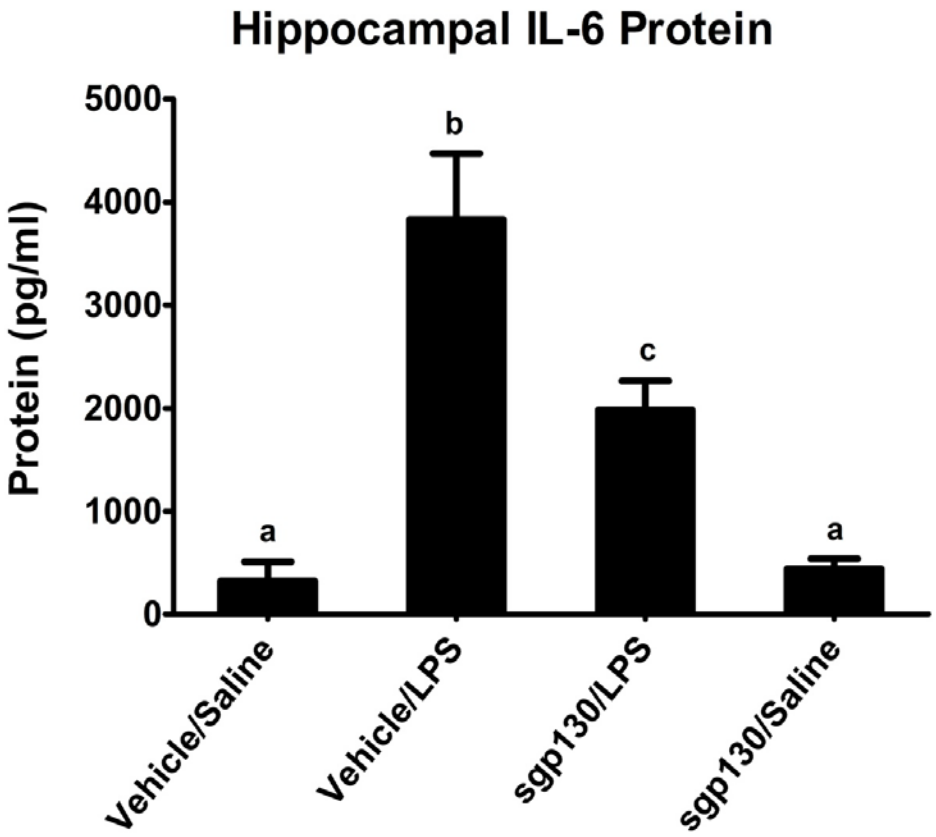


Figure 5.5: Effects of sgp130 on LPS-induced IL-6 in the hippocampus of aged mice. Aged BALB/c mice were injected icv with sgp130 and i.p. with LPS and hippocampal tissue was collected 6 h later for determination of IL-6. The bar graph shows mean IL-6 concentration (± SEM) from 8-9 treatment replicates. Means with different letters are significantly different ($p<0.05$).

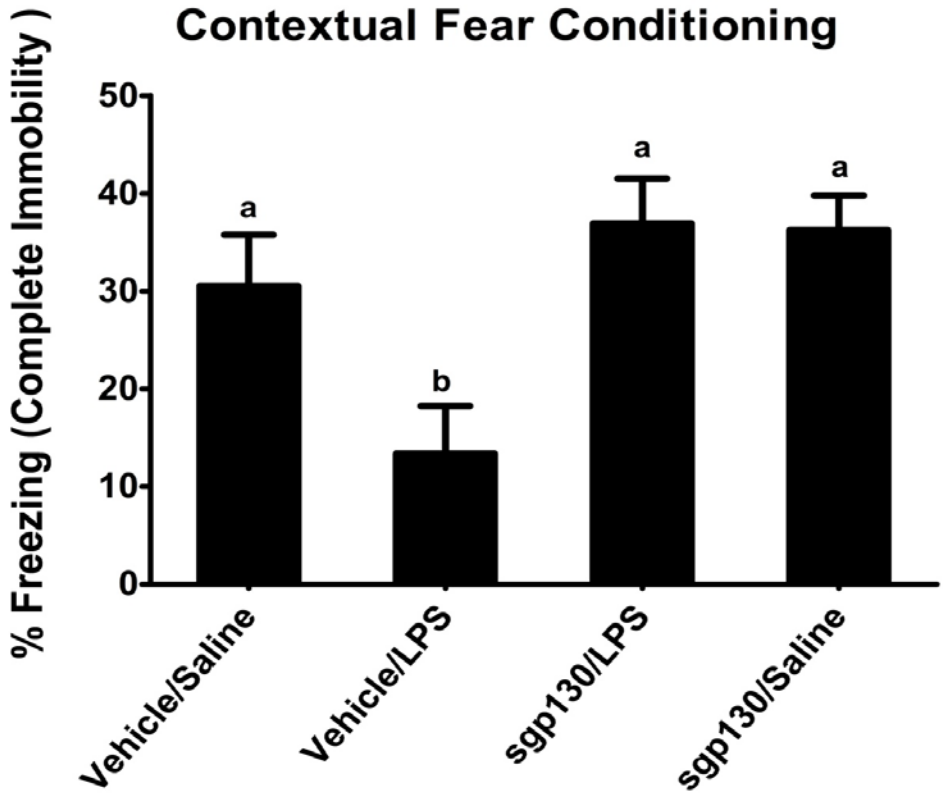


Figure 5.6: Effects of sgp130 on the LPS-induced deficit in contextual fear conditioning in aged mice. Aged BALB/c mice were injected icv with sgp130 and i.p. with LPS immediately after the acquisition phase of contextual fear conditioning. Forty-eight hours later immobility in the retention phase was assessed. Bars represent the mean ± SEM (n = 12-15). Means with different letters are significantly different ($p < 0.05$).

5.7 Literature cited

Aaronson, D.S., Horvath, C.M., 2002. A road map for those who don't know JAK-STAT. Science 296, 1653-1655.

Abraham, J., Jang, S., Godbout, J.P., Chen, J., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging sensitizes mice to behavioral deficits induced by central HIV-1 gp120. Neurobiology of Aging 29, 614-621.

Abraham, J., Johnson, R.W., 2009. Central inhibition of interleukin-1 beta ameliorates sickness behavior in aged mice. Brain Behavior and Immunity 23, 396-401.

Adams, M.M., Smith, T.D., Moga, D., Gallagher, M., Wang, Y., Wolfe, B.B., Rapp, P.R., Morrison, J.H., 2001. Hippocampal dependent learning ability correlates with N-methyl-D-aspartate (NMDA) receptor levels in CA3 neurons of young and aged rats. Journal of Comparative Neurology 432, 230-243.

Balschun, D., Wetzel, W., del Rey, A., Pitossi, F., Schneider, H., Zuschratter, W., Besedovsky, H.O., 2004. Interleukin-6: a cytokine to forget. FASEB Journal 18, 1788.

Barkhausen, T., Tschernig, T., Rosenstiel, P., van Griensven, M., Vonberg, R.P., Dorsch, M., Mueller-Heine, A., Chalaris, A., Scheller, J., Rose-John, S., Seegert, D., Krettek, C., Waetzig, G.H., 2011. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. Critical Care Medicine 39, 1407-1413.

Barrientos, R.M., 2011. Voluntary exercise as an anti-neuroinflammatory therapeutic. Brain Behavior and Immunity 25, 1061-1062.

Barrientos, R.M., Higgins, E.A., Biedenkapp, J.C., Sprunger, D.B., Wright-Hardesty, K.J., Watkins, L.R., Rudy, J.W., Maier, S.F., 2006. Peripheral infection and aging interact to impair hippocampal memory consolidation. Neurobiology of Aging 27, 723-732.

Bellinger, F.P., Madamba, S.G., Campbell, I.L., Siggins, G.R., 1995. Reduced long-term potentiation in the dentate gyrus of transgenic mice with cerebral overexpression of interleukin-6. Neuroscience Letters 198, 95-98.

Bluthe, R.M., Michaud, B., Poli, V., Dantzer, R., 2000. Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. Physiology & Behavior 70, 367-373.

Bruunsgaard, H., Pedersen, M., Pedersen, B.K., 2001. Aging and proinflammatory cytokines. Current Opinion on Hematololy 131, 131 - 136.

Burton, M.D., Sparkman, N.L., Johnson, R.W., 2011. Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. Journal of Neuroinflammation 8, 54.

Chen, J., Buchanan, J.B., Sparkman, N.L., Godbout, J.P., Freund, G.G., Johnson, R.W., 2008. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behavior and Immunity 22, 301-311.

Coles, B., Fielding, C.A., Rose-John, S., Scheller, J., Jones, S.A., O'Donnell, V.B., 2007. Classic interleukin-6 receptor signaling and interleukin-6 trans-signaling differentially control angiotensin II-dependent hypertension, cardiac signal transducer and activator of transcription-3 activation, and vascular hypertrophy in vivo. American Journal of Pathology 171, 315-325.

Cortese, G.P., Barrientos, R.M., Maier, S.F., Patterson, S.L., 2011. Aging and a peripheral immune challenge interact to reduce mature brain-derived neurotrophic factor and activation of TrkB, PLCgamma1, and ERK in hippocampal synaptoneurosomes. Journal of Neuroscience 31, 4274-4279.

Fanselow, M.S., 2000. Contextual fear, gestalt memories, and the hippocampus. Behavioural Brain Research 110, 73-81.

Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W., 2005. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. FASEB Journal 19, 1329.

Godbout, J.P., Johnson, R.W., 2004. Interleukin-6 in the aging brain. Journal of Neuroimmunology 147, 141-144.

Godbout, J.P., Johnson, R.W., 2009. Age and neuroinflammation: A lifetime of psychoneuroimmune consequences. Immunology and Allergy Clinics of North America 29, 321.

Godbout, J.P., Moreau, M., Lestage, J., Chen, J., Sparkman, N.L., Connor, J.O., Castanon, N., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system. Neuropsychopharmacology 33, 2341-2351.

Greenhill, C.J., Rose-John, S., Lissilaa, R., Ferlin, W., Ernst, M., Hertzog, P.J., Mansell, A., Jenkins, B.J., 2011. IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3. Journal of Immunology 186, 1199-1208.

Gruol, D.L., Nelson, T.E., 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Molecular Neurobiology 15, 307-339.

Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F., Graeve, L., 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochemical Journal 334, 297-314.

Hofmann, K.W., Schuh, A.F.S., Saute, J., Townsend, R., Fricke, D., Leke, R., Souza, D.O., Portela, L.V., Chaves, M.L.F., Rieder, C.R.M., 2009. Interleukin-6 serum levels in patients with Parkinson's disease. Neurochemical Research 34, 1401-1404.

Huang, Y., Henry, C.J., Dantzer, R., Johnson, R.W., Godbout, J.P., 2008. Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. Neurobiology of Aging 29, 1744-1753.

Jones, S.A., Richards, P.J., Scheller, J., Rose-John, S., 2005. IL-6 transsignaling: The in vivo consequences. Journal of Interferon and Cytokine Research 25, 241-253.

Jostock, T., Mullberg, J., Ozbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M.F., Rose-John, S., 2001. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. European Journal of Biochemistry 268, 160-167.

Kiecolt-Glaser, J.K., Preacher, K.J., MacCallum, R.C., Atkinson, C., Malarkey, W.B., Glaser, R., 2003. Chronic stress and age-related increases in the proinflammatory cytokine IL-6. Proceedings of the National Academy of Sciences of the United States of America 100, 9090-9095.

Kishimoto, T., Akira, S., Taga, T., 1992. IL-6 receptor and mechanism of signal transduction International Journal of Immunopharmacology 14, 431-438.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., Kohler, G., 1994. Imparied immune and acute-phase responses in interleukin-6 deficient mice. Nature 368, 339-342.

Krzyszton, C.P., Sparkman, N.L., Grant, R.W., Buchanan, J.B., Broussard, S.R., Woods, J., Johnson, R.W., 2008. Exacerbated fatigue and motor deficits in interleukin-10-deficient mice after peripheral immune stimulation. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 295, R1109-R1114.

Licastro, F., Grimaldi, L.M., Bonafe, M., Martina, C., Olivieri, F., Cavallone, L., Giovanietti, S., Masliah, E., Franceschi, C., 2003. Interleukin-6 gene alleles affect the risk of Alzheimer's disease and levels of the cytokine in blood and brain. Neurobiolgy of Aging 24, 921-926.

Maggio, M., Guralnik, J.M., Longo, D.L., Ferrucci, L., 2006. Interleukin-6 in aging and chronic disease: A magnificent pathway. Journals of Gerontology Series 61, 575-584.

McLoughlin, R.M., Jenkins, B.J., Grail, D., Williams, A.S., Fielding, C.A., Parker, C.R., Ernst, M., Topley, N., Jones, S.A., 2005. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proceedings of the National Academy of Sciences of the United States of America 102, 9589-9594.

Muller-Newen, G., Kuster, A., Hemmann, U., Keul, R., Horsten, U., Martens, A., Graeve, L., Wijdenes, J., Heinrich, P.C., 1998. Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses. Journal of Immunology 161, 6347-6355.

Muller, T., Blum-Degen, D., Przuntek, H., Kuhn, W., 1998. Interleukin-6 levels in cerebrospinal fluid inversely correlate to severity of Parkinson's disease. Acta Neurologica Scandinavica 98, 142-144.

Peleg, S., Sananbenesi, F., Zovoilis, A., Burkhardt, S., Bahari-Javan, S., Agis-Balboa, R.C., Cota, P., Wittnam, J.L., Gogol-Doering, A., Opitz, L., Salinas-Riester, G., Dettenhofer, M., Kang, H., Farinelli, L., Chen, W., Fischer, A., 2010. Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328, 753-756.

Pugh, C.R., 2001. The immune system and memory consolidation: a role for the cytokine IL-1 beta. Neuroscience and Biobehavioral Reviews 25, 29-41.

Pugh, C.R., Kumagawa, K., Fleshner, M., Watkins, L.R., Maier, S.F., Rudy, J.W., 1998. Selective effects of peripheral lipopolysaccharide administration on contextual and auditory-cue fear conditioning. Brain Behavior and Immunity 12, 212-229.

Richards, P.J., Nowell, M.A., Horiuchi, S., McLoughlin, R.M., Fielding, C.A., Grau, S., Yamamoto, N., Ehrmann, M., Rose-John, S., Williams, A.S., Topley, N., Jones, S.A., 2006. Functional characterization of a soluble gp130 isoform and its therapeutic capacity in an experimental model of inflammatory arthritis. Arthritis and Rheumatism 54, 1662-1672.

Richwine, A.F., Parkin, A.O., Buchanan, J.B., Chen, J., Markham, J.A., Juraska, J.M., Johnson, R.W., 2008. Architectural changes to CA1 pyramidal neurons in adult and aged mice after peripheral immune stimulation. Psychoneuroendocrinology 33, 1369-1377.

Rosczyk, H.A., Sparkman, N.L., Johnson, R.W., 2008. Neuroinflammation and cognitive function in aged mice following minor surgery. Experimental Gerontology 43, 840-846.

Rose-John, S., Heinrich, P.C., 1994. Soluble receptors for cytokines and growth factors: generation and biological function. Biochemical Journal 300, 281-290.

Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica Et Biophysica Acta-Molecular Cell Research 1813, 878-888.

Schobitz, B., Dekloet, E.R., Sutanto, W., Holsboer, F., 1993. Cellular localization of the interleukin-6 mRNA and interleukin-6 receptor mRNA in rat brain. European Journal of Neuroscience 5, 1426-1435.

Schobitz, B., Pezeshki, G., Pohl, T., Hemmann, U., Heinrich, P.C., Holsboer, F., Reul, J., 1995. Soluble interleukin-6 (IL-6) receptor augments central effects of IL-6 in-vivo. FASEB Journal 9, 659-664.

Sparkman, N.L., Buchanan, J.B., Heyen, J.R.R., Chen, J., Beverly, J.L., Johnson, R.W., 2006. Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. Journal of Neuroscience 26, 10709-10716.

Vallieres, L., Campbell, I.L., Gage, F.H., Sawchenko, P.E., 2002. Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. Journal of Neuroscience 22, 486-492.

Weaver, J.D., Huang, M.H., Albert, M., Harris, T., Rowe, J.W., Seeman, T.E., 2002. Interleukin-6 and risk of cognitive decline - MacArthur studies of successful aging. Neurology 59, 371-378.

Ye, S.M., Johnson, R.W., 1999. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Ye, S.M., Johnson, R.W., 2001. Regulation of interleukin-6 gene expression in brain of aged mice by nuclear factor kappa B. Journal of Neuroimmunology 117, 87-96.

Yirmiya, R., Winocur, G., Goshen, I., 2002. Brain interleukin-1 is involved in spatial memory and passive avoidance conditioning. Neurobiology of Learning and Memory 78, 379-389.

Chapter 6

The selective blockade of the interleukin-6 *trans*-signaling pathway prevents LPS-induced sickness and microglia hyperactivity in the aged

6.1 Abstract

During systemic infection, inflammatory cytokines such as interleukin (IL)-6 are excessively produced in the brain of aged animals and induce infection-related behavioral deficits. However, no studies have examined how pro-inflammatory IL-6 *trans*-signaling on specific cell types is involved in the exaggerated production of IL-6 in the aged brain. Nor the extent to which IL-6 *trans*-signaling affects other markers of neuroinflammation, oxidative stress, adhesion molecules, and behavioral consequences. Therefore, this study investigated the presence of IL-6 signaling subunits, the central effects of soluble gp130 (sgp130), a natural inhibitor of the IL-6 *trans*-signaling pathway, on IL-6 production in microglia and astrocytes of senescent mice, as well as its effects on lipopolysaccharide (LPS)-induced neuroinflammation and behavioral deficits. Here we show that aged microglia have higher surface expression of IL-6 receptor (IL-6R) as well as higher hippocampal gene expression of ADAM17, the enzyme responsible for shedding membrane-bound IL-6R in *trans*-signaling. Additionally, we show that peripheral LPS challenge elicits a hyperactive IL-6 response in both microglia and astrocytes and selective blockade of *trans*-signaling with sgp130 mitigates the exaggerated levels of IL-6 in both microglia and astrocytes. This sgp130-associated inhibition of IL-6 was paralleled by amelioration of the exaggerated and protracted sickness behavior observed in aged animals. Taken together, the results show that microglia and astrocytes are important regulators of the IL-6 *trans*-signaling

response in the aged brain and sgp130 exerts an anti-inflammatory effect by inhibiting the pro-inflammatory arm of IL-6 signaling.

6.2 Introduction

Immune-to-brain communication is important for the appropriate physiological and behavioral response to immune stimuli (Dantzer et al., 1998); this activation stimulates the production of pro-inflammatory cytokines in the central nervous system (Quan, 2008). These pro-inflammatory cytokines are produced by microglia, which are highly dynamic resident macrophages of the brain that are closely associated with astrocytes and neurons (Block et al., 2007). The resulting cytokine production from microglia target neurons to elicit sickness-related behaviors that are adaptive (Kelley et al., 2003).

In aging, this adaptive physiological response becomes maladaptive as there is excessive cytokine production in the brain in response to a peripheral immune stimulus (Godbout et al., 2005b). The exaggerated production of cytokines are associated with behavioral deficits, such as exaggerated sickness behaviors (Abraham and Johnson, 2009a; Godbout et al., 2005b; Huang et al., 2008) and cognitive deficits (Chen et al., 2008). Microglia appear to be the principal cell type responsible for this aberrant cytokine production, as there is a sizeable primed population of microglia seen in the aged (Henry et al., 2009). It is possible that primed microglia play an integral role in mediating a hostile microenvironment that greatly affects astrocyte and neuronal function.

Interleukin-6 production in the CNS has various effects, and evidence suggests it plays a crucial role as a mediator of sickness amongst other behaviors. Studies with IL-

6-deficient animals have implicated that IL-6 production in the hypothalamus is involved in the fever response (Rummel et al., 2006), multiple studies have shown IL-6-deficient animals are resistant to peripheral immune stimulated sickness behaviors (Burton, 2012 ; Nguyen et al., 2011), as well as refractory to LPS-induced deficits in working memory (Sparkman et al., 2006). Moreover, we have recently shown that IL-6 *trans*-signaling in the brain plays a pivotal role in mediating recovery from LPS-induced sickness in adults (Burton et al., 2011). Other studies have supported the notion that IL-6 *trans*-signaling is specifically involved in the pro-inflammatory arm while classic signaling is typically anti-inflammatory or regenerative in nature (Greenhill et al., 2011; Rose-John et al., 2007). While transgenic studies that completely ablate the function of IL-6 gives evidence to its action; we feel it is necessary to develop studies that focus on directly inhibiting the individual signaling pathways, which allows a way to tease out the beneficial functions from the detrimental functions.

IL-6 receptor signaling is facilitated through two related pathways termed classical and *trans*-signaling. The receptor consists of two subunits: the IL-6 receptor-alpha chain (IL-6R), which binds IL-6, and the transmembrane signaling subunit, glycoprotein 130 (gp130), which is the intracellular signal transducer that is constitutively expressed. Classical activation consists of the IL-6 ligand binding to the membrane-bound IL-6R, while *trans*-signaling is the ability of a soluble IL-6R (sIL-6R) to bind IL-6 ligand in the extracellular compartment to form an IL-6/sIL-6R complex. Importantly, the expression pattern of membrane-bound IL-6R is limited to cells of the immune system and sparsely dispersed among other cell types, while gp130 is ubiquitously expressed; therefore IL-6 *trans*-signaling confers IL-6 responsiveness in

any cell type that expresses gp130 (Jones et al., 2005; Kishimoto et al., 1992; Rose-John and Heinrich, 1994).

Both receptor subunits (IL-6R and gp130) are cleaved immediately before the membrane spanning region by alternative splicing or shed by proteolytic enzymes to produce a soluble receptor located in extracellular matrix. While alternative splicing plays a significant role in soluble gp130 (sgp130), it has a much smaller role in the production of sIL-6R. Recent studies on monocytes and other cell lines have shown that two specific members of the a disintegrin and metalloproteinase domain (ADAM) ADAM family of zinc-dependent metalloproteinases, ADAM17 and ADAM10, contribute to the leading mechanism IL-6R shedding to sIL-6R (Matthews et al., 2003)

sIL-6R and sgp130 have varying effects on circulating IL-6. sgp130 acts as an antagonist to the IL-6/sIL-6R complex and prevents the binding of membrane-bound gp130 and further signal transduction (Jostock et al., 2001). Upon binding through either the classical or *trans*-signaling pathway, gp130 dimerizes and autophosphorylates, resulting in the activation of downstream signaling molecules, Janus kinase-1 and 2 (Jak1 and Jak2) and signal transducer and activation of transcription-3 (STAT3). Once STAT3 is activated, it is translocated to the nucleus and binds to enhancer elements of the IL-6 promoter region. It is important to note negative transcriptional regulators of STAT3 activation or suppressors of cytokine signaling (SOCS) proteins 1 and 3 that are present to prevent or curb STAT3 translocation to the nucleus and prevent cytokine-induced signaling.

In recent studies that have begun to tease out the anti- versus pro-inflammatory actions of IL-6, the *trans*-signaling mechanism exhibits its pro-inflammatory actions by

eliciting the production of IL-6 by non-immune cells, which in turn recruit immune cells to the site (Barkhausen et al., 2011; Greenhill et al., 2011; Scheller et al., 2011). The action of IL-6 is heavily dependent on the location of the receptors and the cell types exposed to the cytokine. In the CNS, it has been shown that neurons do not express considerable amounts of IL-6R, however, they do express large amounts of gp130 (Burton et al., 2011; Schobitz et al., 1993), alluding to the importance of IL-6 *trans*-signaling and pro-inflammatory processes in the brain. In aging, our lab has shown that IL-6 is basally upregulated and that microglia are the main cell type responsible for this upregulation (Godbout and Johnson, 2004; Ye and Johnson, 1999b, 2001) . However, how microglia interact with other cells of the CNS and the mechanism of this upregulation is not clearly defined.

In a neurodegenerative model for multiple sclerosis, Linker et., al. found that IL-6 *trans*-signaling modulated the blood brain barrier (BBB), T-cell infiltration and adhesion molecules ICAM-1 and VCAM-1 (Linker et al., 2008), all of which are regulated by astrocytes. Astrocytes are also recognized as a major inducible source of IL-6, however very few studies have focused on astrocytes to ascertain their role in the exaggerated production of IL-6 in aging.

To our knowledge, there are few studies that begin to examine the extent to which peripheral infection influences IL-6 *trans*-signaling, neuroinflammation, and infection-related changes in behavior in the aged brain. Based on data obtained from a previous study (Burton et al., 2011), we believed it was possible that sgp130 would abrogate the exaggerated and prolonged LPS-induced alterations in sickness behavior as well as exaggerated IL-6 levels in aged mice.

The present study investigated cell-specific machinery important for IL-6 activation in adult and aged brain as well as gene expression in the hippocampus, to elude the self-perpetuating mechanism of exaggerated IL-6 production in the aged brain. Further studies assessed the extent to which sgp130 was able to mitigate exaggerated LPS-induced IL-6 production in microglia and astrocytes, as well as augment behavioral deficits in aged mice. Our data show that intracerebroventricular (icv) sgp130 inhibited the onset of sickness behavior, which was paralleled by a decrease in LPS-induced IL-6 gene expression in the hippocampus of sgp130-treated mice. These findings suggest that inhibition of excessive production of IL-6 through its *trans*-signaling pathways during peripheral infection is crucial in preventing behavioral deficits in aged mice.

6.3 Materials and Methods

Animals and Surgery

Adult (3-6 months) and aged (22-24 months) male BALB/c mice were obtained from our in-house breeding colony. The median life span for BALB/c mice is approximately 26 months (Morley and Trainor, 2001), and therefore 3- to 6-month-old (adult) and 20- to 24-month-old (aged) male mice were used. Mice were housed in polypropylene cages and maintained at 21°C under a reverse-phase 12 hour (h) light-dark cycle with *ad libitum* access to water and rodent chow. At the end of each study, mice were examined post mortem for gross signs of disease (e.g., tumors or splenomegaly). Data from mice determined to be unhealthy were excluded from the analysis (< 5%).

Surgery: icv cannulation was performed under aseptic conditions as described previously (Abraham et al., 2008). In brief, mice were deeply anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylazine (100 and 10 mg/kg, respectively) and the surgical site was shaved and sterilized. They were positioned in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA) so that the frontal and parietal bones of the skull were parallel to the surgical platform. An incision roughly 1.5 cm in length was made on the cranium to reveal the bregma and a 26-gauge stainless steel cannula (Plastics One, Roanoke, VA) was placed in the right lateral cerebral ventricle according to predetermined stereotaxic coordinates (lateral 1.6 mm and antero-posterior 1 mm to the bregma, and horizontal 2 mm from the dura mater). The cannula was secured using two adjacent stainless steel screws and cranioplastic cement (Plastics One, Roanoke, VA). A dummy cannula (Plastics One, Roanoke, VA) was inserted in the guide cannula to prevent occlusion and infection. Mice were injected subcutaneously with buprenorphine (0.05 mg/kg) following surgery and then again 8–12 h later to aid with any post-operative discomfort. Mice were provided a minimum of 7 days to recover before any treatment or behavioral test. Accurate placement of the cannula was confirmed by allowing 2 µl of sterile saline to flow via gravity into the lateral ventricle. If cannula placement could not be confirmed by gravity flow the animal was excluded from the study. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Experimental protocols

Mice were routinely handled 1-2 minutes (min) each day for 7 days before experimentation to acclimate them to handling. Animals were injected icv with sterile saline containing 0.1% BSA (vehicle) or 100 ng sgp130 (R&D systems, Minneapolis, MN) dissolved in 2 µl vehicle. At the same time as the icv injection of sgp130, mice were injected i.p. with sterile saline or 0.33 mg/kg BW (10 µg) LPS (serotype 0127:B8, obtained from Sigma, St. Louis, MO).

To measure changes in cytokines, mice not exposed to the behavior paradigms were injected icv with vehicle or sgp130 (100 ng) and i.p. with vehicle or LPS (10 µg), and killed 6 h or 24 h later by CO_2 asphyxiation. The brain was rapidly removed and dissected to obtain hippocampal tissue which was snap frozen in liquid nitrogen and stored at -80^{o}C until later analysis.

Behavioral tests

Locomotor activity: Mice were maintained in their home cage and locomotor activity was video recorded during 5 min intervals using a camera mounted approximately 91.0 cm directly above the center of the cage floor. Behavioral tests were conducted on icv sgp130- and i.p. LPS-treated animals during the dark phase (between 07:00 and 19:00) of the light/dark cycle under infrared lighting to aid video recording. Baseline behavior was taken just before treatment (0 h) and behavior was recorded 4, 6, 8, and 24 h after administration of sgp130 (0 ng or 100 ng icv) and LPS (0 µg or 10µg i.p.). Videos were tracked to by Ethovision (Noldus, Leesburg, VA) software, to record total distance moved. Body weight and food intake were measured at each time point over the 24-h period.

Microglia and Astrocyte isolation

In experiments for flow cytometry, microglia and astrocytes from whole brain were isolated as described previously, with few modifications (Cardona et al., 2006; Henry et al., 2009). Mice were euthanized by CO_2 asphyxiation and whole brains were collected and stored in sterile PBS. Brains were homogenized by passage through a 70 µm cell strainer in Dulbecco's Phosphate Buffered Salt Solution (DPBS) supplemented with 0.2% glucose. Resulting homogenates were centrifuged at 600 × g for 6 min at 10^{o}C. Supernatants were removed and cell pellets were re-suspended in a 70% isotonic Percoll (GE-healthcare, Uppsala, Sweden) supplemented with phenol red (0.01%) at room temperature. A discontinuous Percoll density gradient of 70%, 50%, 35%, and 0% isotonic Percoll was set up. The gradient was centrifuged for 20 min at 2000 × g and microglia were collected from the interphase between the 70% and 50% Percoll layers (Frank et al., 2006), while astrocytes were collected from the interphase between the 50% and 35% Percoll layers (Kozlova and Takenaga, 2005; Park et al., 2012). Cells were washed with DPBS and then re-suspended in PBS- 0.5% BSA/ 0.01% sodium azide solution (flow buffer). The number of viable cells was determined using a hemacytometer and 0.1% trypan blue staining; each isolation yielded approximately 3 $\times 10^{5}$ viable microglia and 4×10^{6} viable astrocytes.

Extracellular and intracellular flow cytometric analysis

Flow cytometric analysis of microglial surface and intracellular markers was performed based on BD Cytofix/Cytoperm Plus fixation/permeabilization protocol (BD biosciences, CA), as described previously, with a few modifications (Henry et al., 2009). In brief, cells isolated by Percoll density gradient were incubated in DMEM (Bio-

Whittaker, Cambrex, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 200 mM glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA), and a solution containing brefeldin A (BD Biosciences, CA) at 37°C in a humidified incubator under 5% CO_2, for 4 h. Cells were then washed in flow buffer and Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, CA). Microglia were first incubated with surface markers, anti-CD11b-Allophycocyanin (APC), anti-CD45-Fluorescein isothiocynate (FITC), and anti-MHC-II-Phycoerythrin (PE) antibodies (eBioscience, CA). Next, both microglia and astrocytes cells were fixed and permeabilized with BD Cytofix/Cytoperm^{TM} solution for 20 min. Cells were washed with BD Perm/Wash^{TM} buffer, re-suspended in BD Perm/Wash^{TM} buffer, astrocytes were incubated with anti-GFAP-Alexa Fluor 488 and anti-IL-6 PE, and microglia were incubated with anti-IL-6-PE (eBioscience, CA) for 30 min. Cells were washed twice in BD Perm/Wash^{TM} buffer and re-suspended in flow buffer. Expression of surface and intracellular antigens was determined using a Becton-Dickinson LSR II Flow Cytometer (Red Oaks, CA). Thirty thousand events were collected, microglia were identified by $CD11b^{+}$ and $CD45^{+low}$ expression (Ford et al., 1995) and astrocytes were identified by $GFAP^{+}$ expression (Raff et al., 1979). Gating was determined based on fluorescently labeled isotype antibodies for PE, FITC, Alexa Fluor 488, and APC (eBioscience, San Diego, CA), and unstained samples as controls. Flow data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Cytokine mRNA measurement by quantitative real-time PCR

Total RNA from hippocampal tissue was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO) A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA)

was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's protocol. Quantitative real-time PCR was performed using the Applied Biosystems (Foster, CA) Assay-on Demand Gene Expression protocol as previously described (Krzyszton et al., 2008). In brief, cDNA was amplified by PCR where a target cDNA (IL-6, Mm00446190_m1;IL-6Rα, Mm00439653_m1; IL-1β, Mm00434228_m1; TNF-α, Mm00443258_m1; IL-10, Mm00439616_m1, ADAM10 Mm00545742_m1; ADAM17 Mm00456428_m1; Icam Mm00516023_m1; Vcam Mm01320970_m1; Socs1Mm00782550_s1; Socs3 Mm00545913_s1; iNOS2 Mm01309902; and a reference cDNA (glucose-3 phosphate dehydrogenase, Mm99999915_g1) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR reactions were performed in triplicate under the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec, and 60^{o}C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, and results are expressed as fold difference.

Statistical analysis

All data were analyzed using the ANOVA routine in Statview and MIXED procedure of the Statistical Analysis System software (SAS Inst., Cary, NC). All data were subjected to a univariate analysis to ensure normality. Locomotor data was subjected to a four-way ANOVA (age × icv × i.p. × time) using repeated measures in which time (0, 4, 6, 8, and 24 h) was a within subjects measure, and age (adult or aged), sgp130 (vehicle or 100 ng/mouse), and LPS (sterile saline or LPS 10 µg/mouse)

were between subjects measures. Weight loss, food intake, microglia and astrocyte data were subjected to a three-way ANOVA (age × icv × i.p.). Gene expression data were subjected to a three-way ANOVA (age × icv × i.p.) or a t-test (age). Post hoc Student's *t* test of least square means was used to determine if treatment means were significantly different from one another ($p<0.05$). All data are presented as mean ± standard error of the mean (SEM).

6.4 Results

IL-6R and gp130 expression on adult vs. aged microglia and astrocytes

Studies in our and other labs have demonstrated an exaggerated brain IL-6 response during infection in aged animals (Godbout and Johnson, 2004; Ye and Johnson, 1999b, 2001). We believe that this dysfunctional IL-6 production in the senescent brain is attributed to dysregulated IL-6 signaling, and becomes a contributing factor to age-related behavioral deficits. Thus, we sought to determine the potential effects of *trans*-signaling on microglia and astrocytes in aging by determining the cellular distribution of IL-6R and gp130 on adult and aged glia. We isolated cell populations of microglia and astrocytes by percoll density gradient, and all cells were subjected to the BD Cytofix/Cytoperm^{TM} fixation/permeabilization protocol. Microglia were stained for CD11b and CD45 and astrocytes were stained for GFAP, and both populations were stained for IL-6R and gp130. Figures 6.1A and B, shows the average percentage of microglia that were CD11b^{+}/IL-6R^{+} and CD11b^{+}/gp130^{+}. Microglia from aged mice had significantly higher expression of IL-6R compared to adult animals ($p<0.05$), while there was no difference in the expression of gp130. Figures 6.2A and 6.2B shows the average percentage of astrocytes that were GFAP^{+}/IL-6R^{+} and

$GFAP^+/gp130^+$. Contrary to microglia data, there was no significant difference of IL-6R expression and there was a visible trend ($p=0.283$) of higher expression of gp130 on astrocytes from aged mice.

Hippocampal tissue was collected 24 h after i.p. sterile saline and assayed for ADAM17 and ADAM10. Aged animals had a significant upregulation of baseline levels of ADAM17 gene expression ($p<0.05$, Figures 6.3A and 6.3B). Taken together, this data suggests the baseline upregulation of IL-6 in the aged is attributed to higher amounts of IL-6R shedding that induce higher levels of IL-6 *trans*-signaling, gene expression, and inflammation in the CNS of aged animals.

sgp130 inhibits LPS-induced sickness behaviors in aged animals

Microglia and astrocytes produce pro-inflammatory cytokines, including IL-6, that induce and facilitate sickness behaviors. Given the results from our recent study (Burton et al., 2011), we investigated the effects of centrally administered sgp130 on LPS-induced sickness behavior in the aged. Adult and aged animals were co-administered *icv* sgp130 and i.p. LPS and locomotor behavior, changes in body weight, and food intake were used as measures of sickness (Figures 6.4, 6.5, 6.6, and 6.7). Locomotor behavior was measured at a baseline (0), 4, 6, 8, and 24 h after injections. As expected, icv vehicle and i.p. LPS treatment decreased locomotor behavior in a time-dependent manner in both adult and aged animals ($p<0.001$). LPS induced modest sickness in adult mice at 4 h, whereby the depression in locomotor behavior returned to baseline level by 24 h post-injection (Figure 6.4).

Also consistent with prior studies, icv vehicle and i.p. LPS treated aged mice (Figure 6.5) exhibited an exaggerated impairment through 24 h post injection (Godbout et al., 2005b). Interestingly, there was a significant age × sgp130 × LPS interaction ($p<0.05$) where LPS-induced depression of locomotor behavior in aged mice was ameliorated by co-administration of icv sgp130, at all time points (Figure 6.5). Important to note, icv sgp130 and i.p. LPS animals, displayed a reduction in locomotor activity animals at the 4 and 6 h time point, which we attributed to LPS-induced IL-1β production. However, when compared to adult animals at 4 and 6 h after LPS injection, aged animals showed a dramatically improved sickness response, compared to *icv* vehicle-treated aged animals that had prolonged depressed locomotor behavior 24 h after LPS (Figure 6.5).

There was no significant difference in icv sgp130-treated adult animals, the animals remained sick until they recovered 24 h post injection as in the icv vehicle treated animals. This data is differs from social exploratory data, in which sgp130 improves recovery by 8 h in adult animals. It appears as if there is motivational input of some sickness behaviors where differences are observed.

Additionally, sgp130 had significant mitigating effects on LPS-induced reductions in food intake and weight loss (Figures 6.6A and B) in aged animals, but not adult animals. While sgp130 did not return either behavior to baseline, the exaggeration of sickness seen in aging was ameliorated indicating that the response the maladaptive response was curbed to adaptive levels. These data suggest that the upregulated IL-6 *trans*-signaling pathway is more sensitive in aged animals for the onset of sickness, while it is important for maintaining sickness behavior in adult animals.

sgp130-mediated LPS-induced microglia production of IL-6 and priming

To determine the microglial distribution and signaling capacity of IL-6 production in the CNS, we determined intracellular IL-6 protein expression in microglia isolated from adult and aged mice 6 h after icv sgp130 and i.p. LPS injection. Figure 6.8 shows the average percentage of microglia from adult and aged mice stained with anti-CD11b-APC and intracellular anti-IL-6-PE. As expected, peripheral LPS injection increased the percentage of IL-6^+ microglia ($CD11b^+$/IL-16^+) in both age groups (Figure 6.8). In a comparison between LPS-treated adult and aged mice, there was a significant difference in the percentage of IL-6^+ microglia ($CD11b^+$/IL-6^+) in aged mice ($p<0.05$), an observation consistent with earlier findings from our lab (Ye and Johnson, 1999a). Figure 6.8 goes on to show that icv sgp130 attenuates IL-6 production from aged microglia, taking the levels down similar to that of adult expression levels. While sgp130 did not completely abrogate the exaggerated expression of IL-6, the maladaptive levels seen in aging were curtailed and mirror the observations seen in the sickness behavior, to an adaptive response.

Previous studies have shown that IL-6 induces MHC-II on microglia and *trans*-signaling potentiates this affect (Burton, 2012). Therefore, we determined if sgp130 augmented increased MHC-II surface expression on microglia isolated from aged mice. Isolated microglia from adult and aged brains, were stained with antibodies for CD11b, CD45, and MHC-II, and then analyzed by flow cytometry. Figure 6.9 is the average percentage of $CD11b^+$/MHC-II^+ cells, and shows consistency with previous that microglia isolated from aged mice had significantly higher MHC-II expression

(CD11b^{+}/MHC II^{+}) compared to the adult microglia. There was no significant age × sgp130 × LPS interaction, although there was a slight decrease of MHC-II in the sgp130- treated aged group. A possible explanation for this result is the exposure of sgp130 was not sufficient to have a significant effect. Taken together, these cytokine protein data indicate that peripheral immune stimulation caused microglia from aged mice to become hyper-responsive, and is associated with increased production of IL-6, and sgp130 mitigates the hyperactivity.

sgp130-mediated LPS-induced astrocyte production of IL-6

Previous studies have shown that numbers and markers of astrocyte activation do not increase with aging (Ye and Johnson, 1999a), but to date no one has determined astrocyte involvement in cytokine production during peripheral infection and aging. To determine the astrocyte distribution and signaling capacity of IL-6 production in the CNS, we determined intracellular protein expression of IL-6 in astrocytes isolated from adult and aged mice 6 h after icv sgp130 and i.p. LPS injection. Figure 6.10 shows the average percentage of astrocytes from adult and aged mice stained with anti-GFAP-Alexa Fluor-488 and intracellular anti-IL-6-PE. Peripheral LPS injection increased the percentage of IL-6^{+} astrocytes (GFAP^{+}/IL-16^{+}) in both age groups (Fig. 6.10). Similar to microglia, there was a significantly higher percentage of IL-6^{+} astrocytes (GFAP^{+}/IL-6^{+}) in aged mice compared to adult mice ($p<0.05$). icv sgp130 attenuates IL-6 production from both adult and aged astrocytes, a stark contrast to the microglia data in which only the aged microglia were affected. These data indicate that LPS-induced astrocyte-

derived IL-6 is modulated from microglia and may or may not directly affect sickness behaviors.

Exaggerated IL-6 and MHC-II gene expression is modified by sgp130

Increased mRNA levels of inflammatory glial markers have been detected in the aged brain (Dilger and Johnson, 2008). The action of sgp130 is anticipated at the IL-6 receptor level, but it is important to assess if it immediately affects other cytokines, regulators/modulators of cytokine expression, or the BBB. It is important to assay mRNA transcripts as it can indicate a change that would be seen quicker than a change in protein levels. In order to assay gene expression the hippocampus was used as a representation of the global neuroinflammatory response and various markers of inflammation (IL-1β, IL-6,), oxidative stress (iNOS2), cytokine signal regulation (SOCS1 and SOCS3), cytokine modulation (ADAM10 and ADAM17), and markers of BBB integrity (ICAM-1 and VCAM-1) were measured.

A number of studies correlate exaggerated IL-6 mRNA levels with alterations in behavioral process (Bellinger et al., 1995; Gruol and Nelson, 1997; Maggio et al., 2006; Weaver et al., 2002). With the robust effects of sgp130 inhibition of LPS-induced sickness behavior, IL-6 protein in microglia and astrocytes in aged animals, we were interested in the effect of IL-6 *trans*-signaling inhibition on IL-6 gene expression. Adult and aged mice received co-administration of sgp130 (icv) and LPS (i.p.) and hippocampal tissue was harvested 6 h after. Figure 6.11A shows data similar to protein levels. There was a significant age × sgp130 × LPS interaction ($p<0.05$), whereby sgp130 significantly decreased the basal and LPS-induced expression of IL-6 in the aged brain, and showing a decreasing trend in the adult.

Based on the findings of sgp130-mediated MHC-II protein expression, we measured gene expression, as timing dictates that gene expression would be first affected in altering MHC-II. Interestingly, sgp130 significantly decreased the basal age-associated upregulation MHC-II gene expression ($p<0.05$, Figure 6.11B). Taken together, these results further validate that sgp130 is working at the receptor level and IL-6 *trans*-signaling is involved in microglia priming.

sgp130 does not affect oxidative stress, cytokine regulators, modulators or BBB

sgp130-mediated decrease of LPS-induced IL-6 mRNA in the hippocampus may have other consequences that could account for the alterations in sickness behavior and microglial hyperactivity. Figures 6.12 – 6.15 show, IL-1β and iNOS2, ADAM17 and ADAM10, SOCS1 and SOCS3, and ICAM-1 and VCAM-I, respectively. Overall, there was no significant sgp130 × age × LPS interaction for any of the above genes. However, worth mentioning, icv sgp130 decreased i.p. LPS-induced IL-1β (Figure 6.12A) and VCAM-1 (Figure 6.15B) expression of aged animals to similar expression levels of adult animals, but not significantly different than icv vehicle treated age-matched animals. This could indicate the well documented IL-1β and IL-6 interaction and bi-directional influence (Tosato and Jones, 1990). Also noteworthy is the age-dependent basal upregulation of adhesion molecules ICAM-1 and VCAM-1, perhaps indicative of more traffic across the BBB during aging, or potentially to compensate for a loss of protein (Mooradian, 1988). While expression of these genes is not altered at this time point, the initial window of sgp130 effectiveness may manifest downstream changes. Taken together, these results show that sgp130-related changes in LPS-

induced sickness behavior are paralleled by sgp130-associated changes of IL-6 in cells of the brain.

6.5 Discussion

The bi-directional communication between the periphery and the brain is essential to mount the appropriate response to an immune stimulus (Dantzer et al., 1998). However, in aging this physiological response turns to a pathological state as there is excessive cytokine production in the brain in response to a peripheral immune stimulus (Godbout and Johnson, 2009). This pathological production of cytokines are coupled with an increase of behavioral deficits (Dilger and Johnson, 2008). Excessive pro-inflammatory IL-6 in the brain plays a critical role in the development of these infection-related alterations in sickness behavior and cognition (Bluthe et al., 2000; Burton and Johnson, 2011; Maggio et al., 2006). Here, we extended our previous work on IL-6 production in the aged brain, and begin to elucidate a mechanism for exaggerated levels in the brain. First we showed that aged microglia expressed a higher level of IL-6R (Figure 6.1A) and the aged brain expressed higher ADAM17 sheddase (Figure 6.3A), than adult animals. This data suggests that microglia are primarily responsible for shedding IL-6R and inducing the IL-6 *trans*-signaling in the microenvironment of the CNS. Similar observations have been documented in models of peripheral inflammation and colon cancer, the researchers found upregulated IL-6R on T-cells and an increase in ADAM17, alluding to IL-6 *trans*-signaling is involved in immune cell regulation and disease (Becker et al., 2004).

The idea is that age-dependent upregulation of IL-6 *trans*-signaling is facilitated through a receptor stimulated STAT3-dependent mechanism of feed-forward IL-6

production in the brain of aged animals (Burton and Johnson, 2011) before and during peripheral stimulation. We therefore investigated the effects of icv sgp130 and hypothesized that, given the role of IL-6 in neuroinflammatory responses; sgp130 would attenuate LPS-induced sickness behavior and IL-6 production. sgp130 was effective in inhibiting the onset of LPS-induced depression of locomotor behavior as well as LPS-induced anorexia and reduction in food intake in aged mice. Moreover, we showed that icv sgp130 inhibited LPS-induced sickness in aged animals, and these sgp130-associated effects were paralleled by reduced mRNA transcript in the hippocampus, and protein levels of IL-6 in microglia and astrocytes.

Consistent with previous studies, a reduction in brain IL-6 did not completely prevent the initial induction of LPS-induced sickness behavior seen at 4 h post-injection in adult animals, (Abraham and Johnson, 2009a; Berg et al., 2004; Godbout et al., 2004; Godbout et al., 2005a; Henry et al., 2008), and this is the case because LPS is such a potent immune stimulus. Moreover, the amelioration of the exaggerated LPS-induced behavioral deficits seen in aging has been observed in various nutritional and pharmacological interventions and our observations are consistent with previous studies (Abraham and Johnson, 2009a; Berg et al., 2005; Burton and Johnson, 2011; Richwine et al., 2005). These data suggest that aged animals are more sensitive to changes in pro-inflammatory mediators, foreseeable going from maladaptive to adaptive levels.

This inhibition of LPS-induced sickness in aged mice may be of particular importance when considering conditions where an exaggerated response is elicited during a primed inflammatory state, such as in overexpressing transgenic animals (Campbell et al., 1993; Heyser et al., 1997), prion disease (Combrinck et al., 2002), and

neurodegenerative diseases (Betmouni et al., 1996; Cunningham et al., 2005; Du et al., 2001; Hofmann et al., 2009; Perry et al., 2003). We have previously demonstrated that aged animals display an exaggerated neuroinflammatory and sickness behavior response after activation of the peripheral immune system (Godbout et al., 2005b), and it appears that primed microglia are responsible for this exacerbated phenotype (Godbout et al., 2004; Sparkman and Johnson, 2008; Ye and Johnson, 1999b). Current data beings to uncover that astrocytes also have a role in IL-6 production in the aged system.

Successful activation of the IL-6/IL-6R complex results in enhanced production of IL-6 and blocking this pathway with sgp130 will reduce the production of IL-6 and other proteins that interact with IL-6. The present study demonstrates that the exaggerated levels of LPS-induced IL-6 in aging are associated with IL-6 *trans*-signaling and higher levels of the sheddase ADAM17. However at the time point of 6 h after sgp130 and LPS treatment, gene expression of cytokine regulators, modulators, oxidative stress, and BBB were left essentially unaltered. IL-6 is predominately the protein that is affected in this system proving the importance of the protein.

A recent study found an IL-6 *trans*-signaling-mediated crosstalk between the TLR-4 and STAT3 pathway to reveal *trans*-signaling is implicated in pro-inflammatory action versus classical signaling (Greenhill et al., 2011). Using sgp130 allowed us to investigate the effects of IL-6 after LPS treatment, while leaving all other cytokines unaffected.

We and other groups have shown that anti-inflammatory interventions are able to ameliorate the exaggerated cytokine as well as the maladaptive response that results

from peripheral infection in aged individuals (Abraham and Johnson, 2009a, b; Berg et al., 2005; Henry et al., 2008; Jang et al., 2010; Richwine et al., 2005). These data agree with other studies using sgp130 to inhibit IL-6 signaling in peripheral models of inflammation, such as arthritis, peritonitis, and colitis (Coles et al., 2007; McLoughlin et al., 2005; Richards et al., 2006). To our knowledge, this is the first study to report that co-administration with sgp130 attenuates LPS-induced exaggerations in sickness and IL-6 protein production in microglia and astrocytes in aged animals. Studies have highlighted the potential therapeutic role of sgp130 in treating peripheral inflammatory conditions; as well as to suppress the severity of EAE and modulate neutrophil trafficking (Atreya et al., 2000; Chen et al., 2004; Fielding et al., 2008; Linker et al., 2008). The current study is the first to extend the body of literature and show the effectiveness of sgp130 in inhibiting IL-6 signaling in cells of the aged CNS. Taken together, the present study suggests that the use of sgp130 as an inhibitor of the IL-6 pathway in an array of inflammatory conditions, from peripheral to neurodegenerative disease, mitigates IL-6 expression and has a beneficial effect on sickness-related behavioral responses.

6.6 Figures

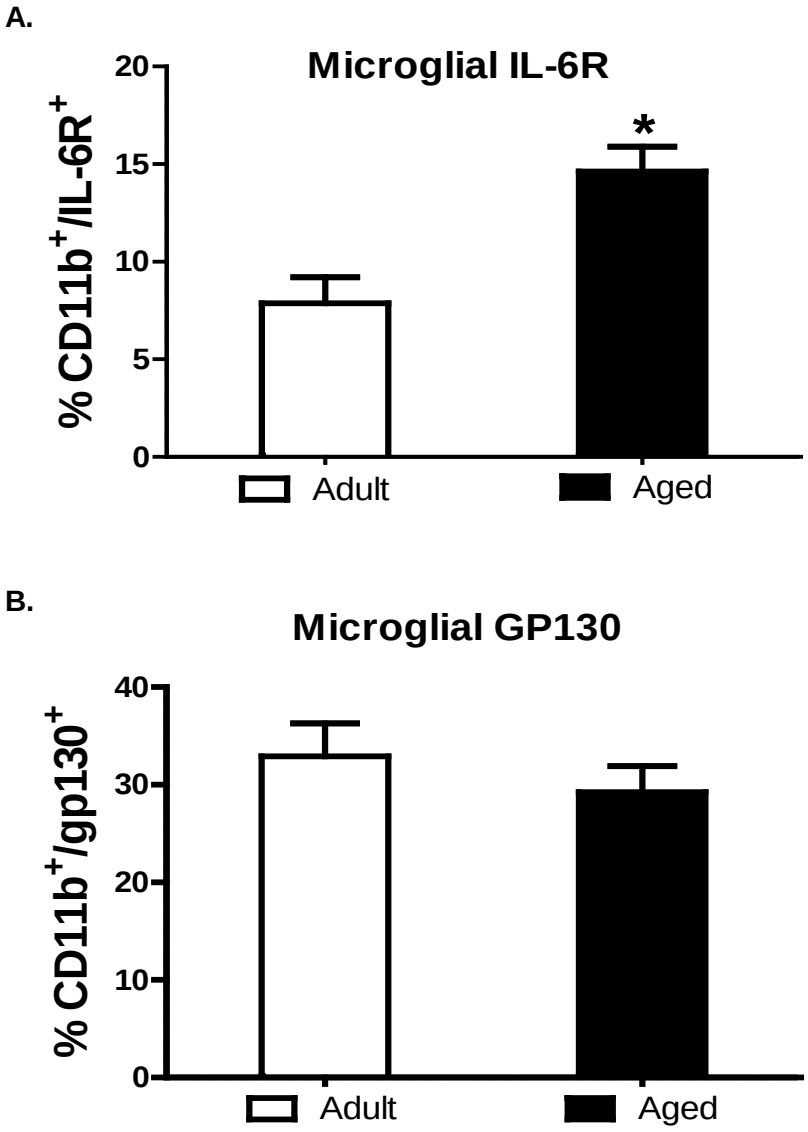


Figure 6.1: Differential expression of IL-6 receptor and gp130 on microglia isolated from adult and aged mice. Average percentage of cells that were A) $CD11b^+/IL\text{-}6R^+$ B) and $CD11b^+/gp130^+$. Cell surface markers were assessed for CD11b, CD45, and IL-6R or gp130 expression; compared with isotype and unstained controls. Bars represent the means ± SEM (n = 8). Means marked with a * are significantly different from each other (p<0.05).

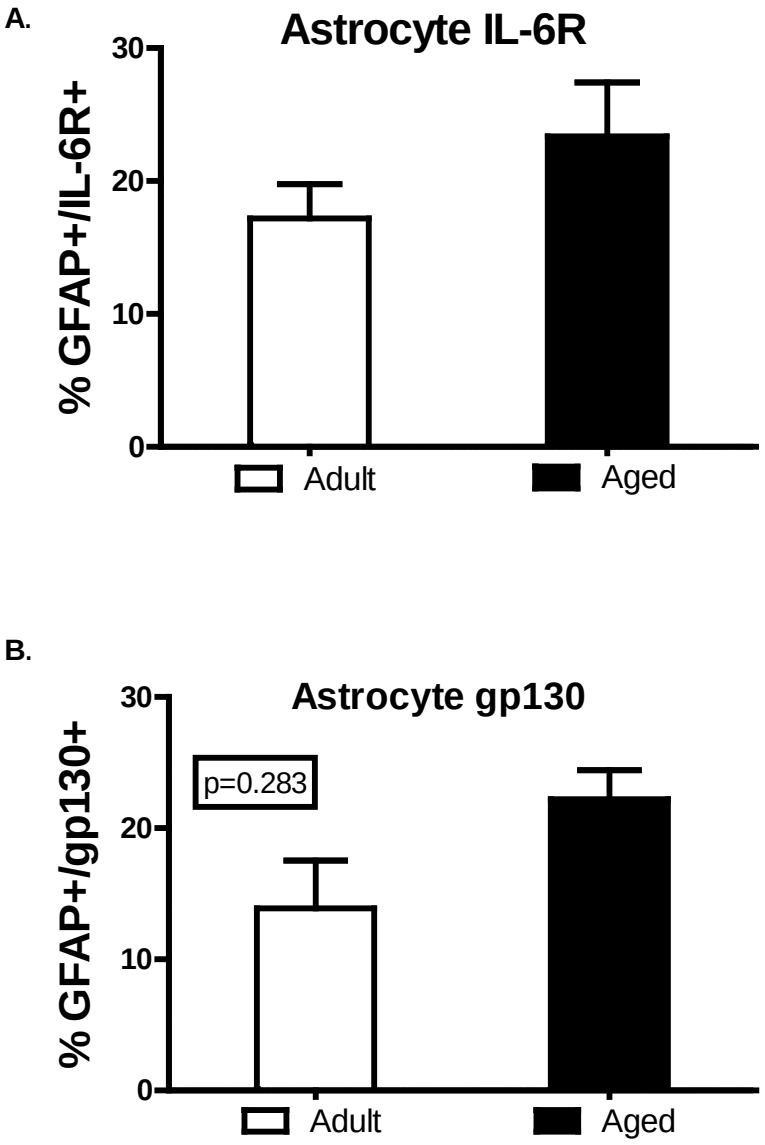
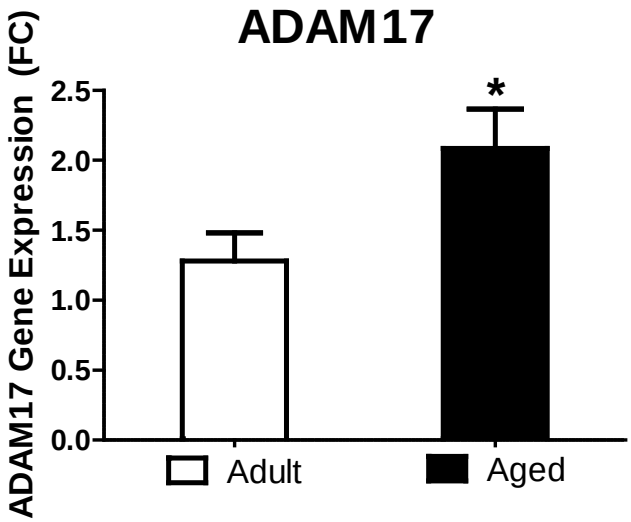


Figure 6.2: Differential expression of IL-6 receptor and gp130 on astrocytes isolated from adult and aged mice. Average percentage of cells that were A) $GFAP^+/IL\text{-}6R^+$ B) and $GFAP^+/gp130^+$. Cell surface markers were assessed for GFAP and IL-6R or gp130 expression; compared with isotype and unstained controls. Bars represent the means ± SEM (n = 8). Means marked with a * are significantly different from each other (p<0.05).

A.



B.

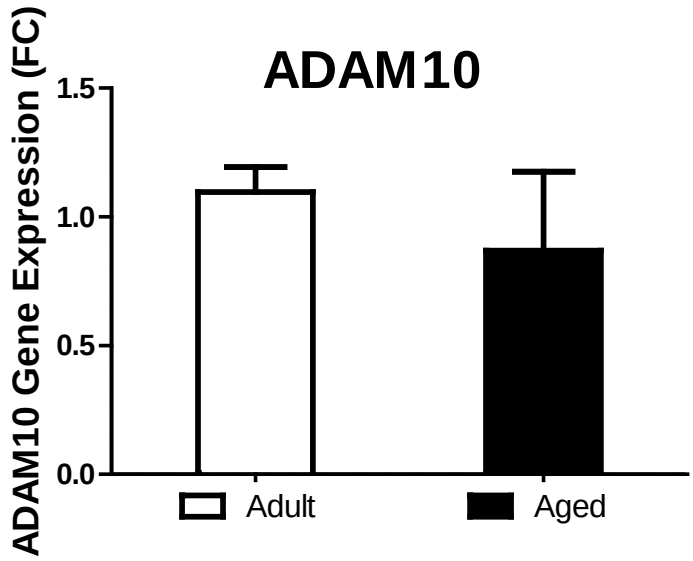


Figure 6.3: Hippocampal gene expression of IL-6 receptor sheddases ADAM17 and ADAM10 from adult and aged mice. Hippocampal tissue was collected 24 h after i.p. sterile saline and assayed for A) ADAM17 and B) ADAM10. Aged animals had a significant upregulation of baseline levels of ADAM17 gene expression. Bars represent the mean ± SEM (n = 8-9) Means with * are statistically different from each other ($p<0.05$).

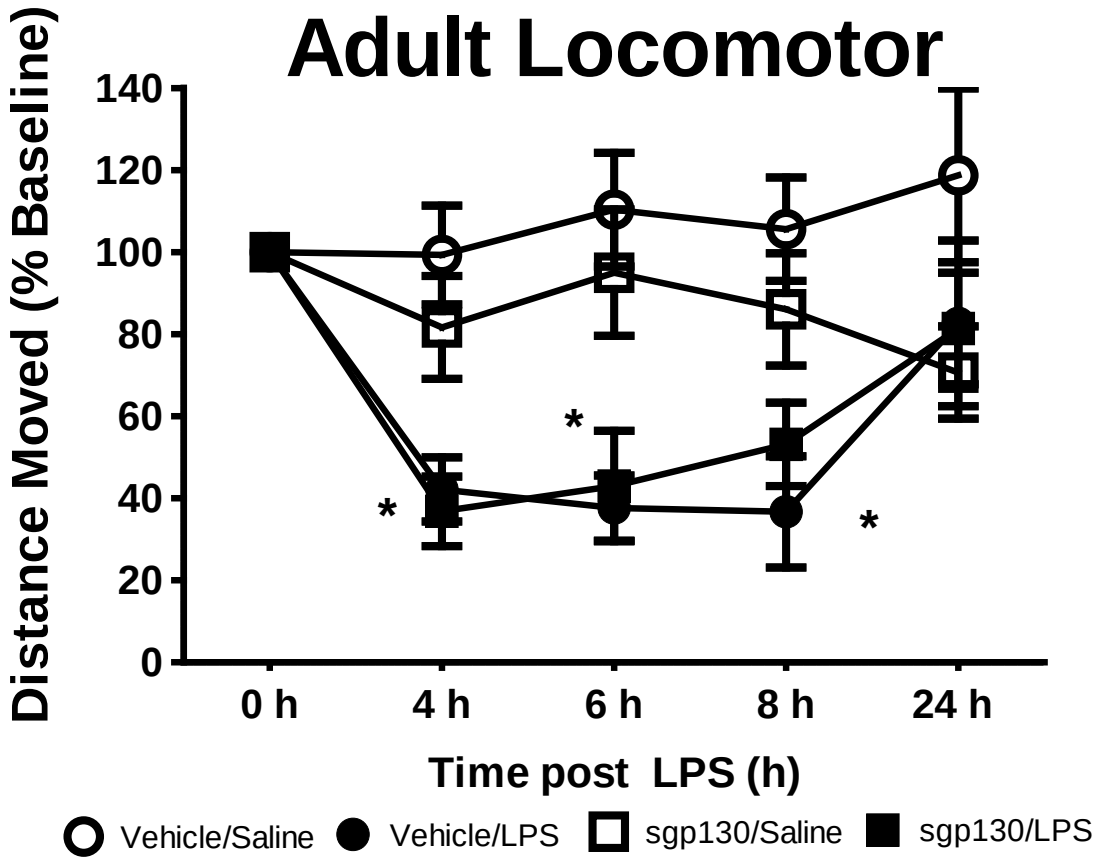


Figure 6.4: sgp130-mediated LPS-induced sickness behavior in adult animals. Mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg). Spontaneous locomotor baseline behavior was measured before injections (0) and at 4, 6, 8, and 24 h post-injection. Bars represent the mean ± SEM (n = 10-11) Means with * are statistically different ($p<0.05$) from saline controls.

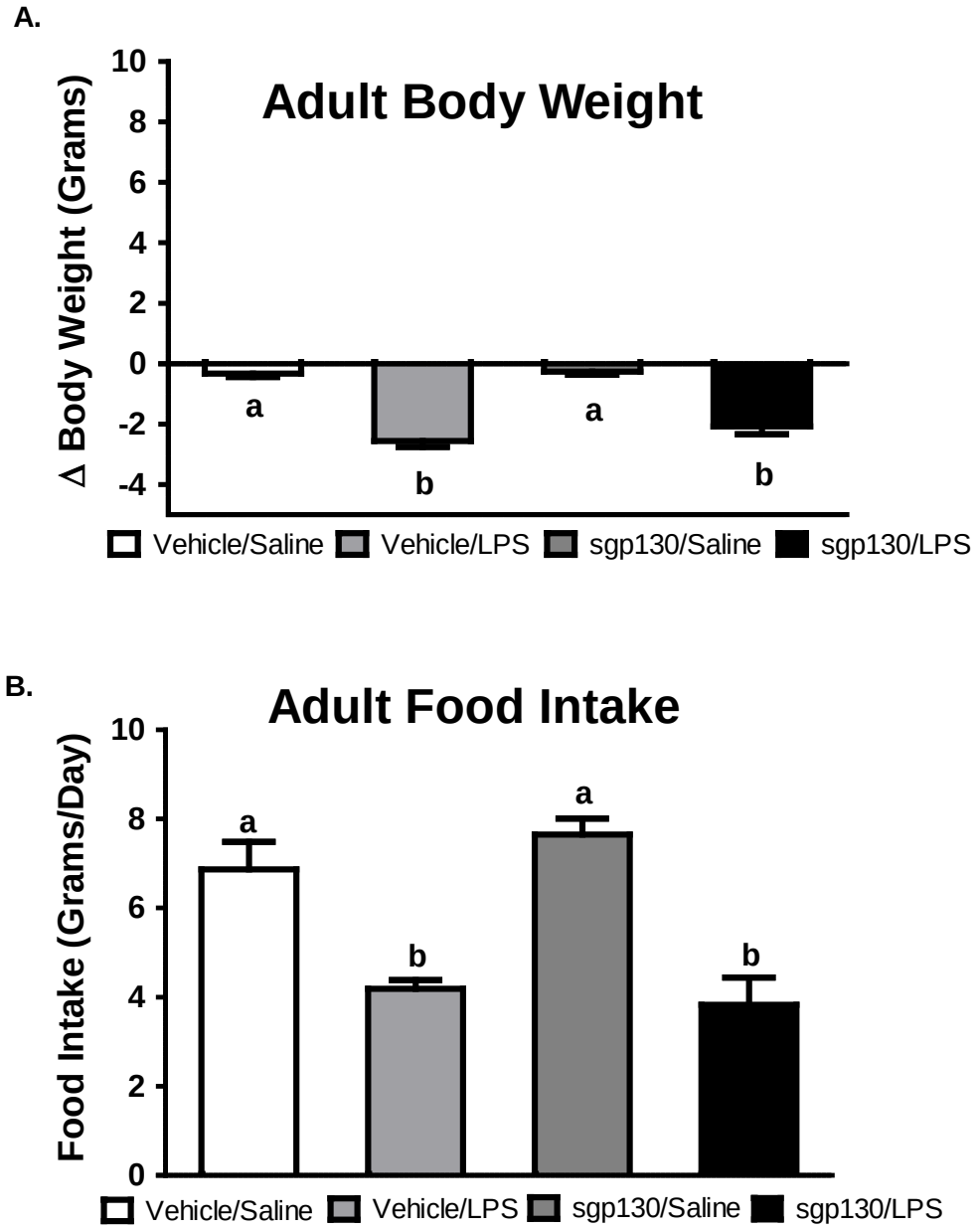


Figure 6.5: sgp130-mediated LPS-induced sickness behavior in adult animals. Mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg). A) Body and B) food weight was measured before LPS injection and at 24 h post-injection. Bars represent the mean ± SEM (n = 10-11) Means with * are statistically different ($p<0.05$) from saline controls.

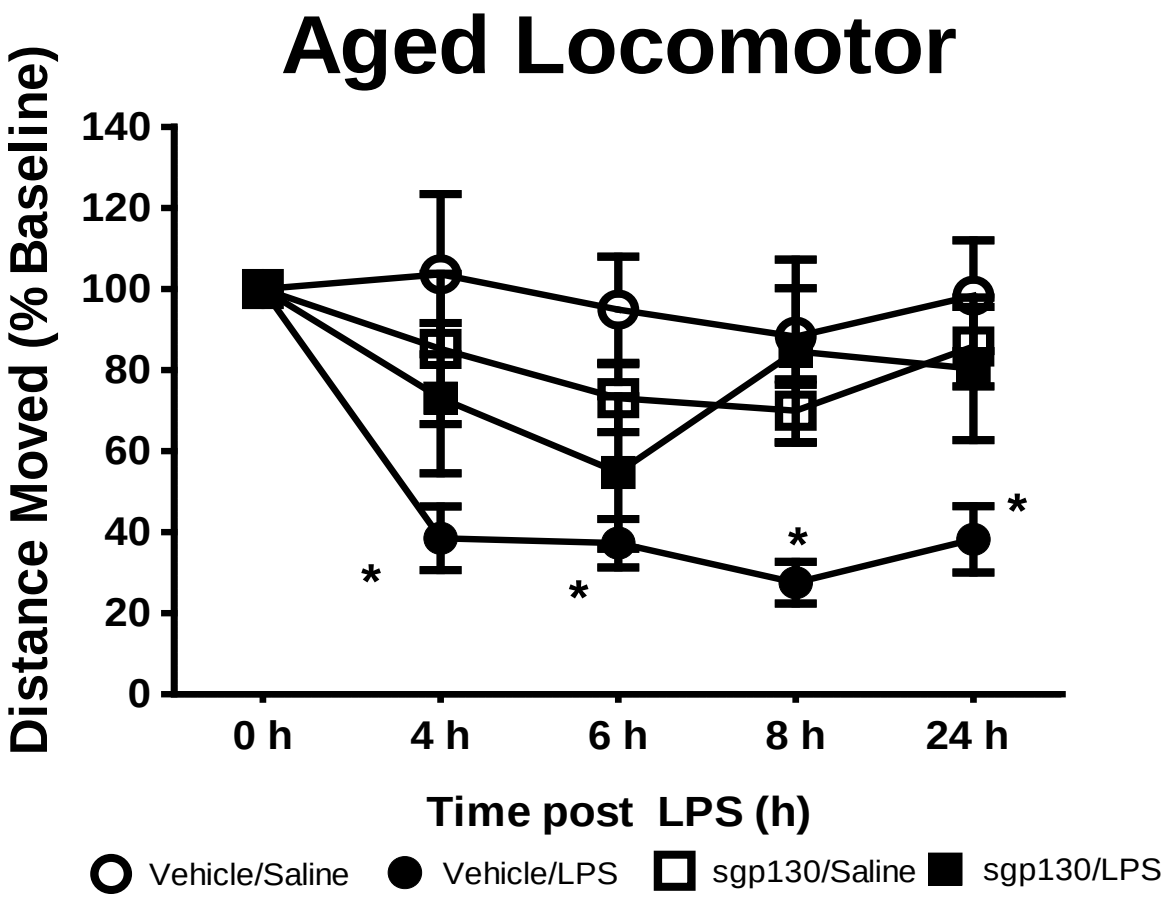


Figure 6.6: sgp130-mediated LPS-induced sickness behavior in aged animals. Mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg). Spontaneous locomotor baseline behavior was measured before injections (0) and at 4, 6, 8, and 24 h post-injection. Bars represent the mean ± SEM (n = 10-12). Means with different letters are statistically different ($p<0.05$) from vehicle/saline controls.

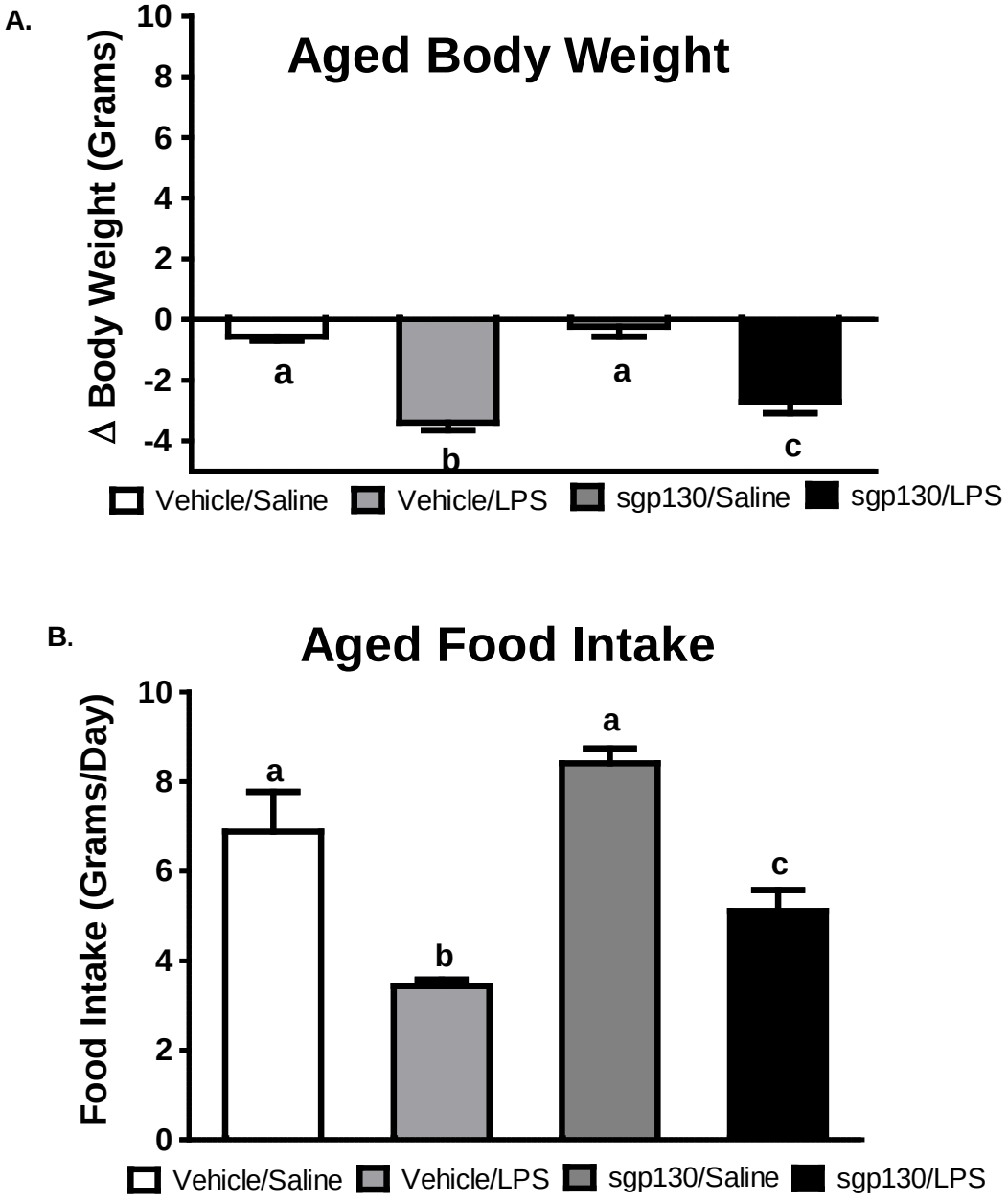


Figure 6.7: sgp130-mediated LPS-induced sickness behavior in aged animals. Mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg). A) Body and B) food weight was measured before LPS injection and at 24 h post-injection. Bars represent the mean ± SEM (n = 10-11). Means with different letters are statistically different ($p<0.05$) from vehicle/saline controls.

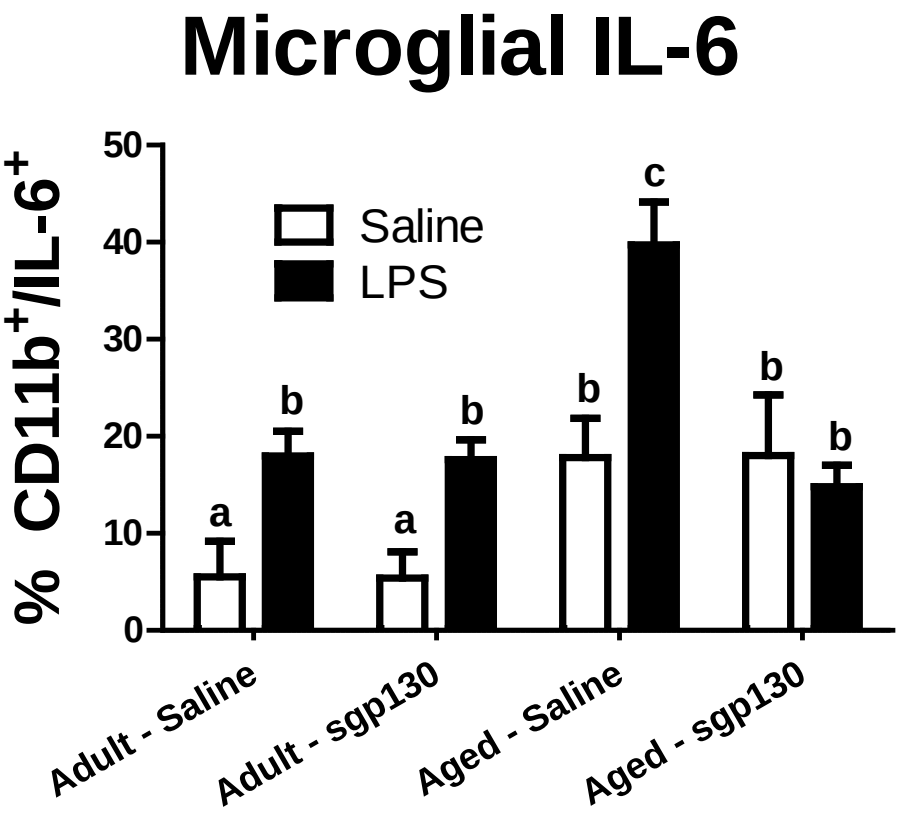


Figure 6.8: sgp130 augments LPS-induced microglial IL-6 production in the aged. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and microglia were isolated 6 h later by percoll density gradient. Cells were subjected to the BD Cytofix/Cytoperm^{TM} fixation/permeabilization protocol and stained with anti-CD11b-APC, anti-CD45-FITC, and anti-IL-6-PE. Average percentage of cells that were CD11b^{+}/IL-6^{+}. Bars represent the means ± SEM (*n* =7-8). Means with different letters are statistically different ($p<0.05$) from each other.

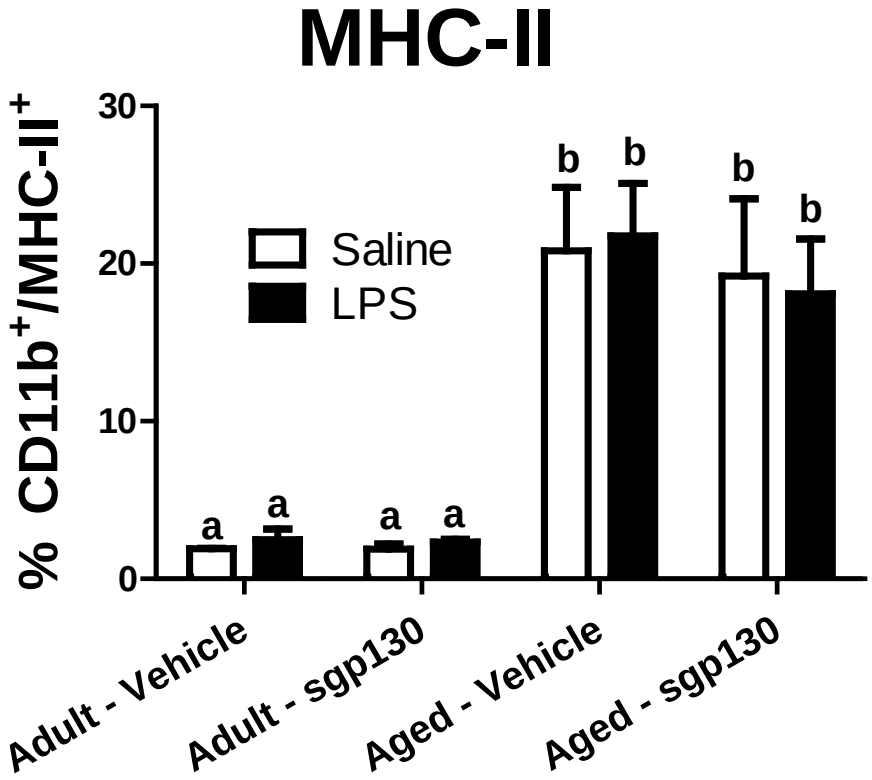


Figure 6.9: Microglial MHC-II expression on adult and aged mice. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and microglia were isolated 6 h later by percoll density gradient. Cells were subjected to the BD Cytofix/Cytoperm^{TM} fixation/permeabilization protocol and stained with anti-CD11b-APC, anti-CD45-FITC, and anti-MHC-II-PE. Average percentage of cells that were $CD11b^+$/MHC-II^+. Bars represent the means ± SEM (*n* =7-8). Means with different letters are statistically different ($p<0.05$) from each other.

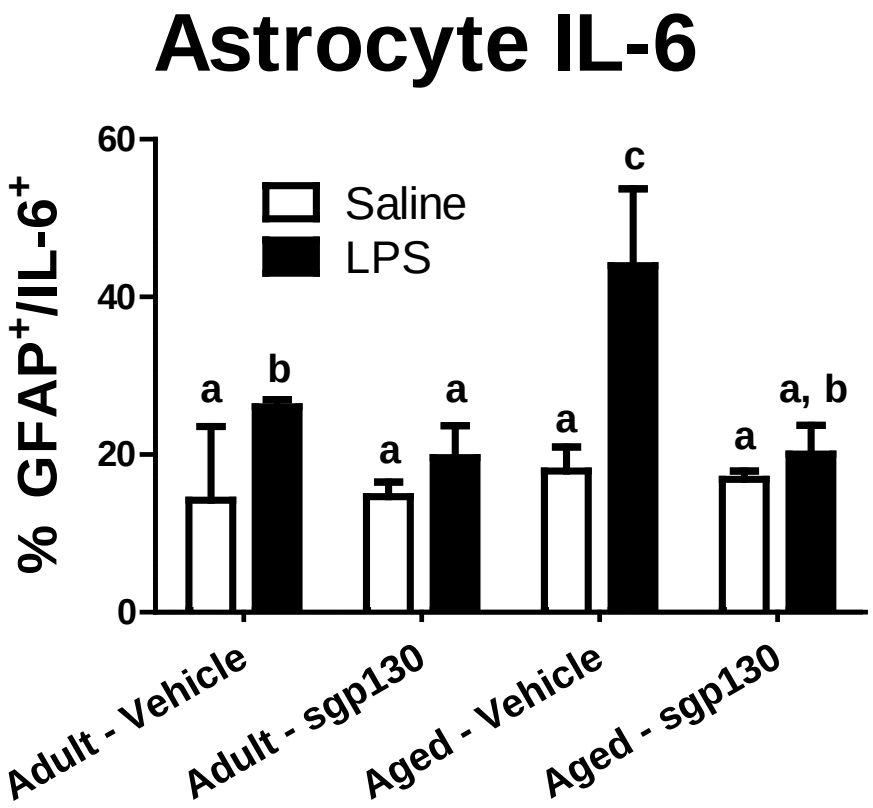


Figure 6.10: sgp130 augments LPS-induced astrocyte l IL-6 production in the aged. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and astrocytes were isolated 6 h later by percoll density gradient. Cells were subjected to the BD Cytofix/Cytoperm^{TM} fixation/permeabilization protocol and stained with anti-GFAP-Alexa Fluor 488 and anti-IL-6-PE. Average percentage of cells that were GFAP^{+}/IL-6^{+}. Bars represent the means ± SEM (*n* =7-8). Means with different letters are statistically different ($p<0.05$) from each other.

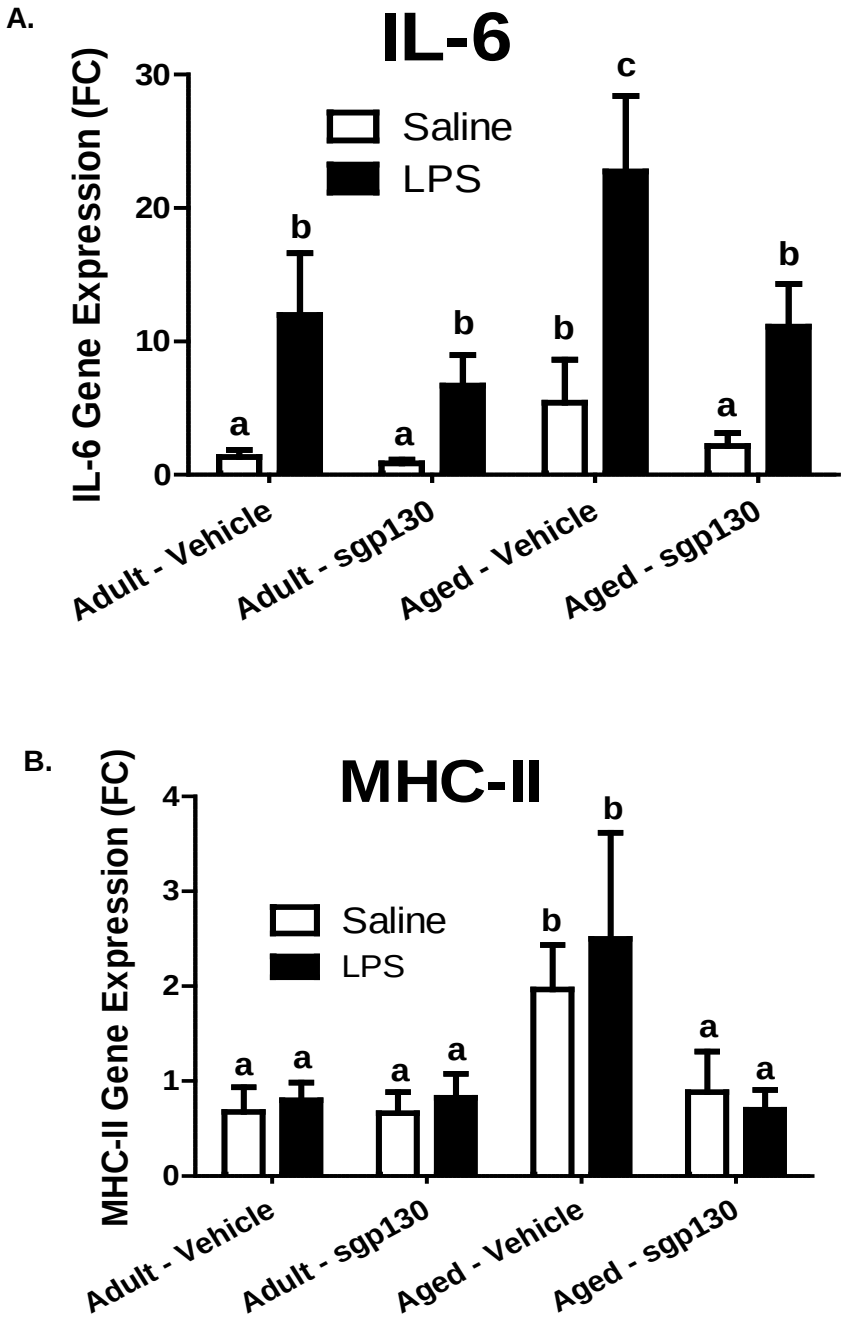


Figure 6.11: sgp130-mediated LPS-induced hippocampal gene expression of IL-6 and MHC-II from adult and aged mice. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and hippocampal tissue was collected 8 h later and assayed for A) IL-6 and B) MHC-II. Bars represent the mean ± SEM (n = 7-8) Means with different letters are statistically different ($p<0.05$) from each other.

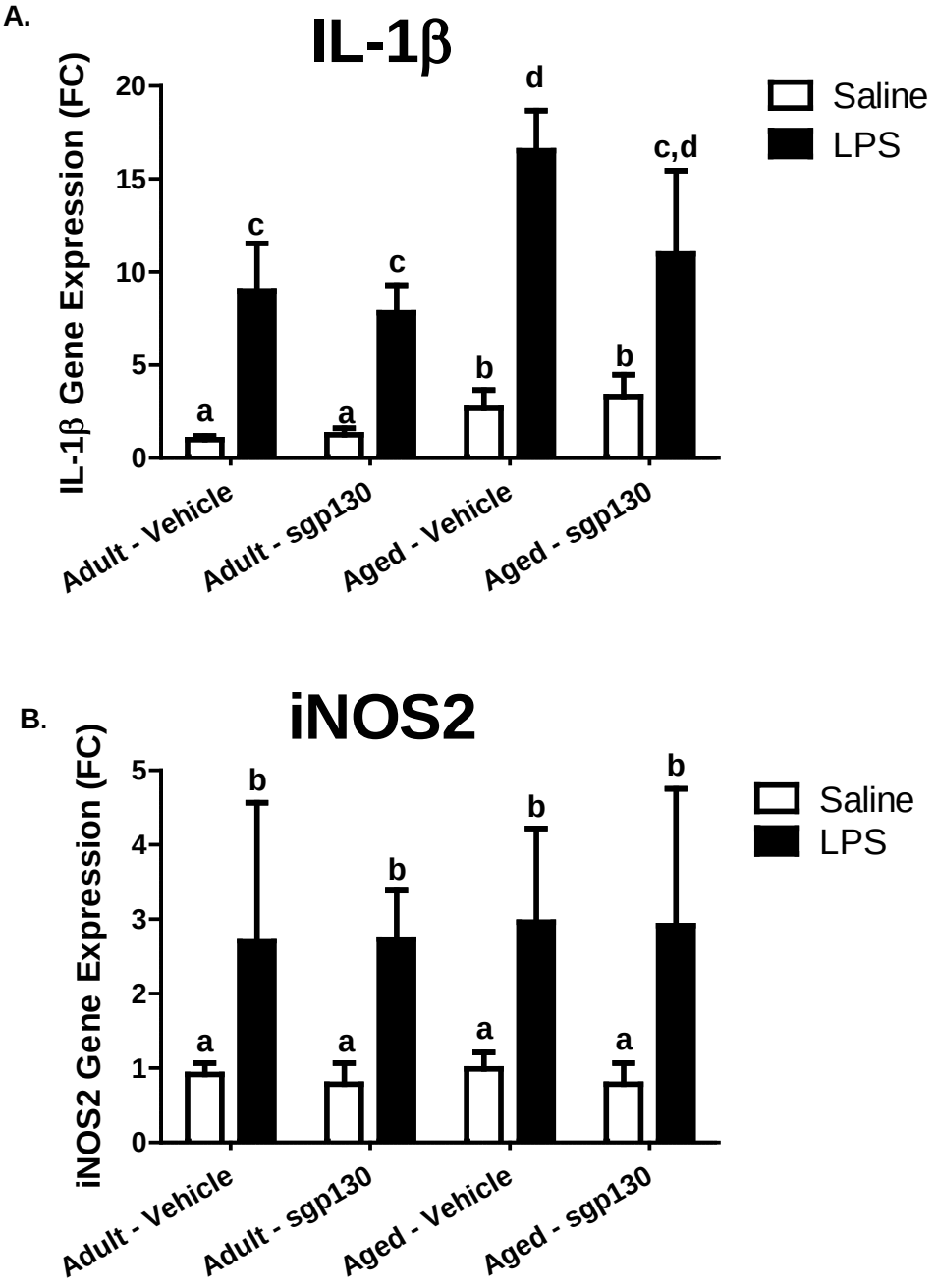


Figure 6.12: sgp130-mediated LPS-induced hippocampal gene expression of IL-1β and iNOS2 from adult and aged mice. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and hippocampal tissue was collected 8 h later and assayed for A) IL-1β and B) iNOS2. Bars represent the mean ± SEM (n = 7-8) Means with different letters are statistically different ($p<0.05$) from each other.

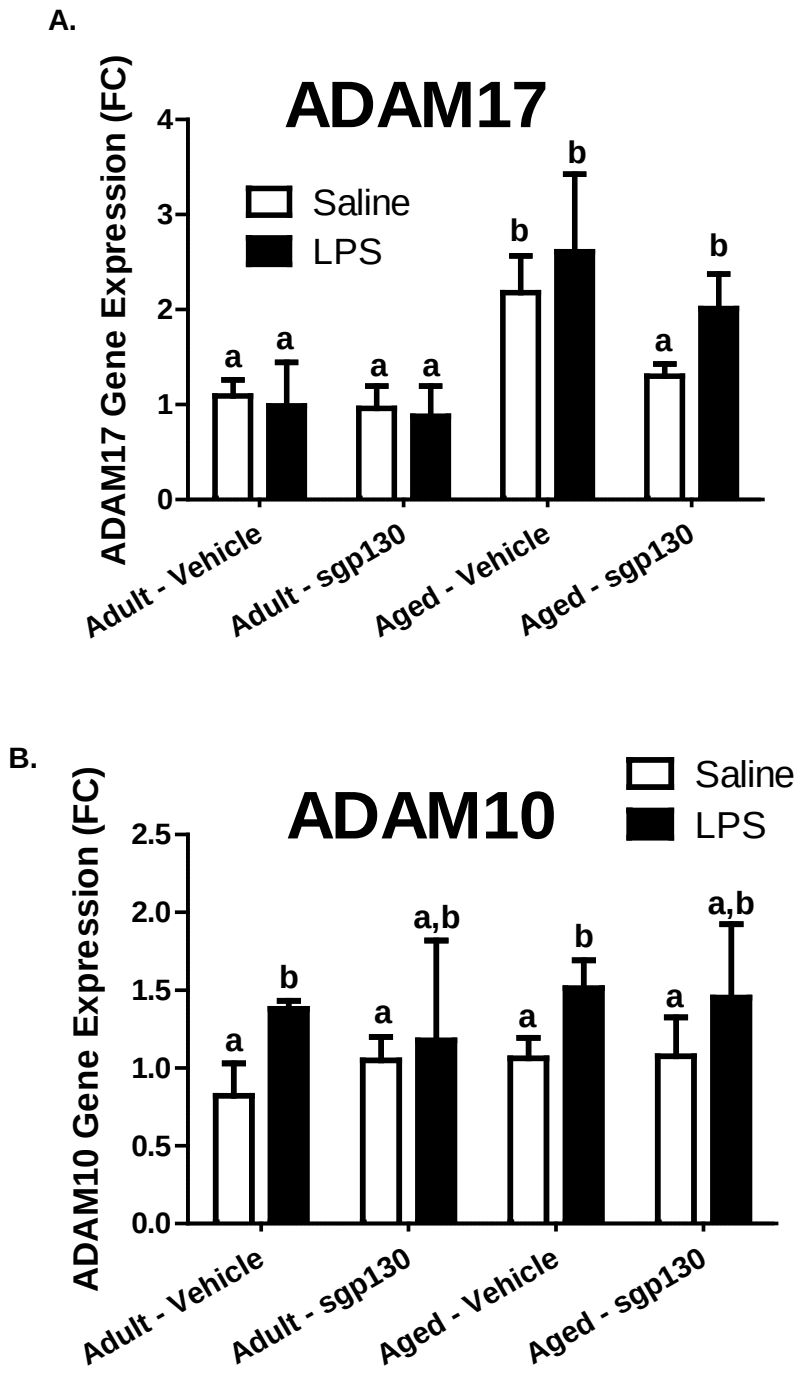


Figure 6.13: sgp130-mediated LPS-induced hippocampal gene expression of ADAM17 and ADAM10 from adult and aged mice. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and hippocampal tissue was collected 8 h later and assayed for A) ADAM17 and B) ADAM10. Bars represent the mean ± SEM (n = 7-8) Means with different letters are statistically different ($p<0.05$) from each other.

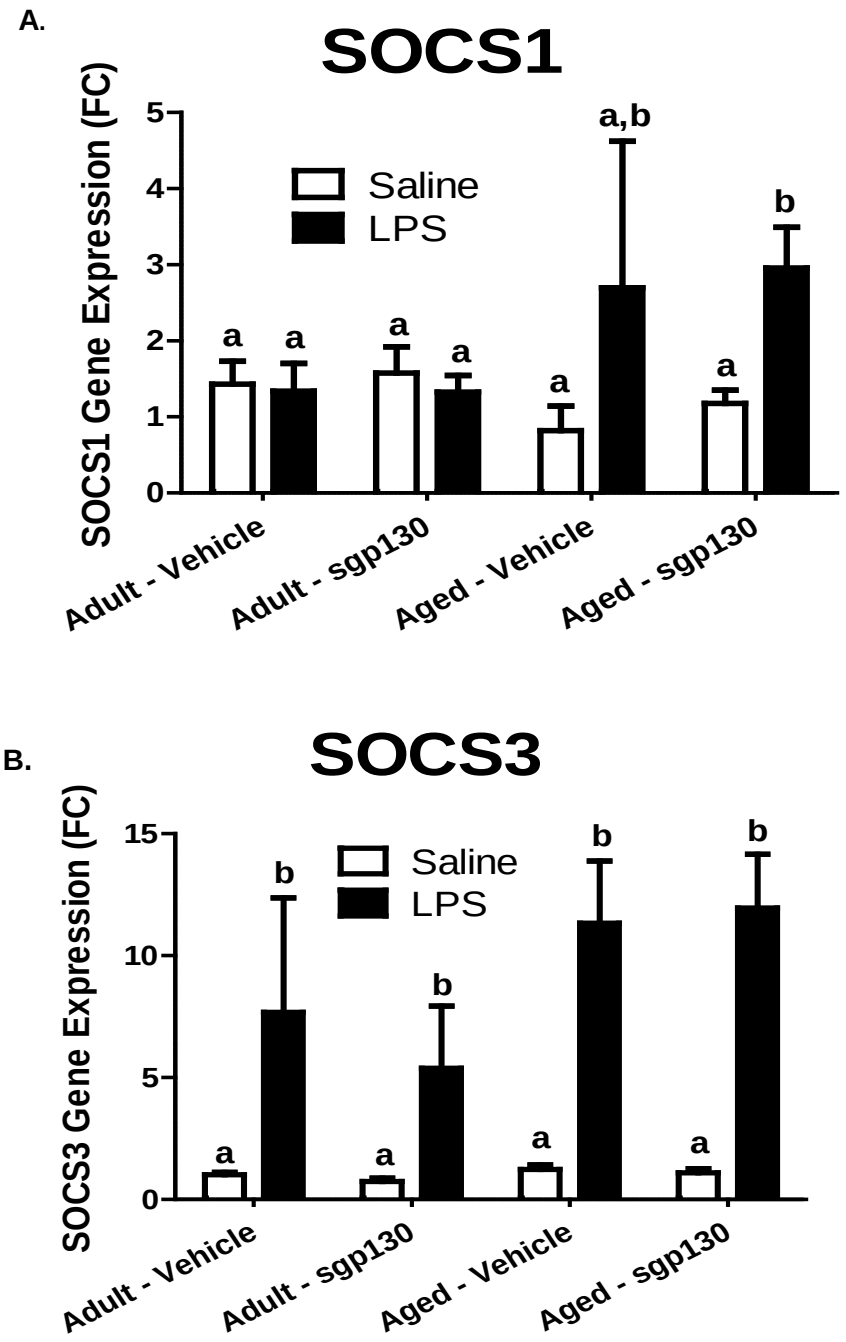


Figure 6.14: sgp130-mediated LPS-induced hippocampal gene expression of SOCS1 and SOCS3 from adult and aged mice. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and hippocampal tissue was collected 8 h later and assayed for A) SOCS1 and B) SOCS3. Bars represent the mean ± SEM (n = 7-8) Means with different letters are statistically different ($p<0.05$) from each other.

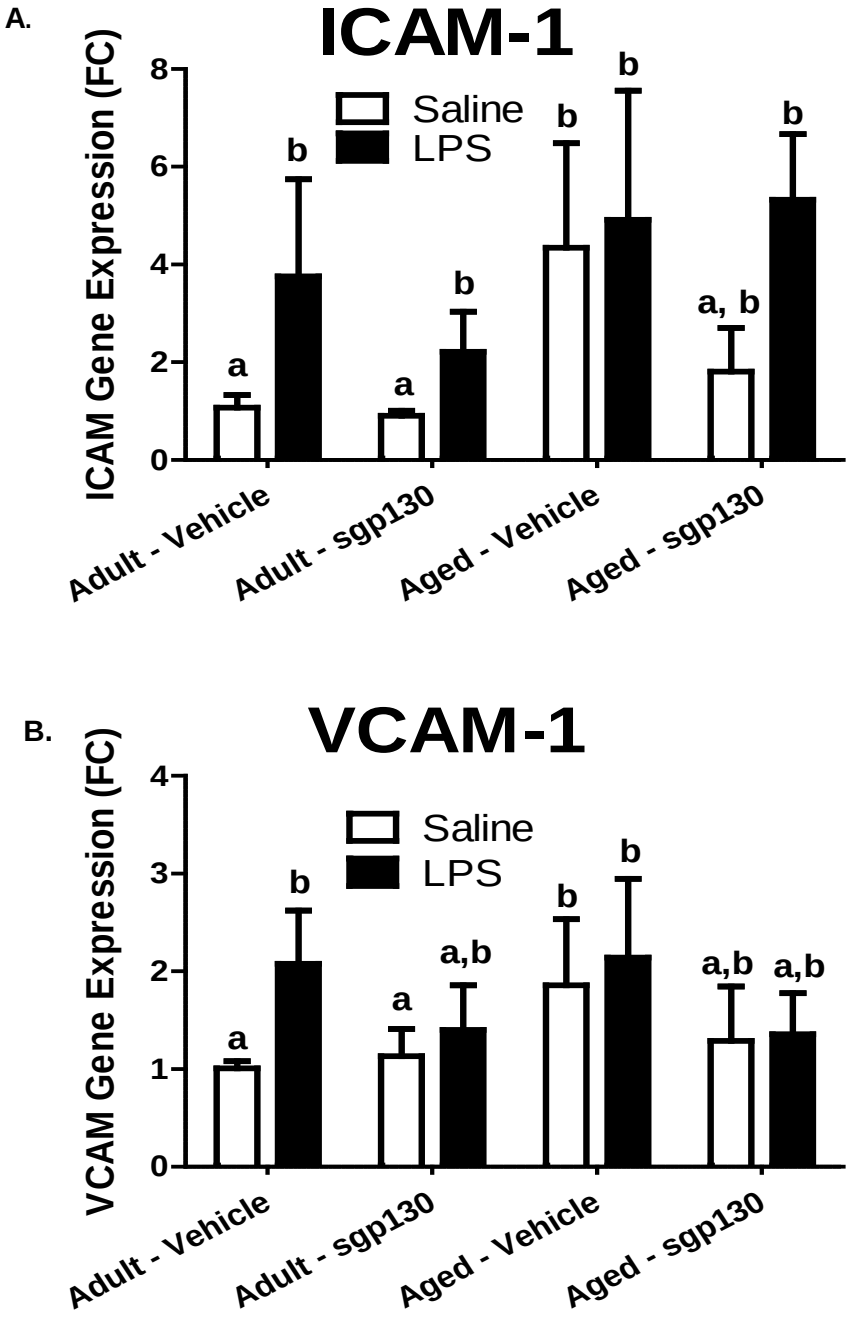


Figure 6.15: sgp130-mediated LPS-induced hippocampal gene expression of ICAM-1 and VCAM-1 from adult and aged mice. Adult and aged mice were injected icv with vehicle or sgp130 (100ng) and i.p. with sterile saline or LPS (10 µg) and hippocampal tissue was collected 8 h later and assayed for A) ICAM-1 and B) VCAM-1. Bars represent the mean ± SEM (n = 7-8) Means with different letters are statistically different ($p<0.05$) from each other.

6.7 Literature cited

Abraham, J., Jang, S., Godbout, J.P., Chen, J., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging sensitizes mice to behavioral deficits induced by central HIV-1 gp120. Neurobiology of Aging 29, 614-621.

Abraham, J., Johnson, R.W., 2009a. Central inhibition of interleukin-1 beta ameliorates sickness behavior in aged mice. Brain Behavior and Immunity 23, 396-401.

Abraham, J., Johnson, R.W., 2009b. Consuming a diet supplemented with resveratrol reduced infection-related neuroinflammation and deficits in working memory in aged mice. Rejuvenation Research 12, 445-453.

Atreya, R., Finotto, S., Mudter, J., Mullberg, J., Jostock, T., Holtmann, M., Kishimoto, T., Galle, P.R., Rose-John, S., Neurath, M.F., 2000. Blockade of IL-6 transsignaling abrogates established experimental colitis in mice by suppression of T cell resistance against apoptosis. Gastroenterology 118, 4803.

Barkhausen, T., Tschernig, T., Rosenstiel, P., van Griensven, M., Vonberg, R.P., Dorsch, M., Mueller-Heine, A., Chalaris, A., Scheller, J., Rose-John, S., Seegert, D., Krettek, C., Waetzig, G.H., 2011. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. Critical Care Medicine 39, 1407-1413.

Becker, C., Fantini, M.C., Schramm, C., Lehr, H.A., Wirtz, S., Nikolaev, A., Burg, J., Strand, S., Kiesslich, R., Huber, S., Ito, H., Nishimoto, N., Yoshizaki, K., Kishimoto, T., Galle, P.R., Blessing, M., Rose-John, S., Neurath, M.F., 2004. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. Immunity 21, 491-501.

Bellinger, F.P., Madamba, S.G., Campbell, I.L., Siggins, G.R., 1995. Reduced long-term potentiation in the dentate gyrus of transgenic mice with cerebral overexpression of interleukin-6. Neuroscience Letters 198, 95-98.

Berg, B.M., Godbout, J.P., Chen, J., Kelley, K.W., Johnson, R.W., 2005. Alpha-tocopherol and selenium facilitate recovery from lipopolysaccharide-induced sickness in aged mice. Journal of Nutrition 135, 1157-1163.

Berg, B.M., Godbout, J.P., Kelley, K.W., Johnson, R.W., 2004. Alpha-Tocopherol attenuates lipopolysaccharide-induced sickness behavior in mice. Brain Behavior and Immunity 18, 149-157.

Betmouni, S., Perry, V.H., Gordon, J.L., 1996. Evidence for an early inflammatory response in the central nervous system of mice with scrapie. Neuroscience 74, 1-5.

Block, M.L., Zecca, L., Hong, J.S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature Reviews: Neuroscience 8, 57-69.

Bluthe, R.M., Michaud, B., Poli, V., Dantzer, R., 2000. Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. Physiology & Behavior 70, 367-373.

Burton, M.D., Johnson, R.W., 2011. Interleukin-6 trans-signaling in the senescent mouse brain is involved in infection-related deficits in contextual fear conditioning. Brain Behavior and Immunity 26, 738-738.

Burton, M.D., Johnson, R.W., 2012 Interleukin-6 is involved in microglia priming and the exaggerated LPS-induced sickness response in aged mice. (in prep).

Burton, M.D., Sparkman, N.L., Johnson, R.W., 2011. Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. Journal of Neuroinflammation 8, 54.

Campbell, I.L., Abraham, C.R., Masliah, E., Kemper, P., Inglis, J.D., Oldstone, M.B.A., Mucke, L., 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin-6. Proceedings of the National Academy of Sciences of the United States of America 90, 10061-10065.

Cardona, A.E., Huang, D., Sasse, M.E., Ransohoff, R.M., 2006. Isolation of murine microglial cells for RNA analysis or flow cytometry. Nature Protocols 1, 1947-1951.

Chen, J., Buchanan, J.B., Sparkman, N.L., Godbout, J.P., Freund, G.G., Johnson, R.W., 2008. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behavior and Immunity 22, 301-311.

Chen, Q., Wang, W.C., Bruce, R., Li, H., Schleider, D.M., Mulbury, M.J., Bain, M.D., Wallace, P.K., Baumann, H., Evans, S.S., 2004. Central role of IL-6 receptor signal-transducing chain gp130 in activation of L-selectin adhesion by fever-range thermal stress. Immunity 20, 59-70.

Coles, B., Fielding, C.A., Rose-John, S., Scheller, J., Jones, S.A., O'Donnell, V.B., 2007. Classic interleukin-6 receptor signaling and interleukin-6 trans-signaling differentially control angiotensin II-dependent hypertension, cardiac signal transducer and activator of transcription-3 activation, and vascular hypertrophy in vivo. American Journal of Pathology 171, 315-325.

Combrinck, M.I., Perry, V.H., Cunningham, C., 2002. Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease. Neuroscience 112, 7-11.

Cunningham, C., Wilcockson, D.C., Campion, S., Lunnon, K., Perry, V.H., 2005. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. Journal of Neuroscience 25, 9275-9284.

Dantzer, R., Bluthe, R.M., Gheusi, G., Cremona, S., Laye, S., Parnet, P., Kelley, K.W., 1998. Molecular basis of sickness behavior. In: Kluger, M.J., Bartfai, T., Dinarello, C.A. (Eds.), Molecular Mechanisms of Fever, pp. 132-138.

Dilger, R.N., Johnson, R.W., 2008. Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. Journal of Leukocyte Biology 84, 932-939.

Du, Y.S., Ma, Z.Z., Lin, S.Z., Dodel, R.C., Gao, F., Bales, K.R., Triarhou, L.C., Chernet, E., Perry, K.W., Nelson, D.L.G., Luecke, S., Phebus, L.A., Bymaster, F.P., Paul, S.M., 2001. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 98, 14669-14674.

Fielding, C.A., McLoughlin, R.M., McLeod, L., Colmont, C.S., Najdovska, M., Grail, D., Ernst, M., Jones, S.A., Topley, N., Jenkins, B.J., 2008. IL-6 regulates neutrophil

trafficking during acute inflammation via STAT3. Journal of Immunology 181, 2189-2195.

Ford, A.L., Goodsall, A.L., Hickey, W.F., Sedgwick, J.D., 1995. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. Journal of Immunology 154, 4309-4321.

Frank, M.G., Wieseler-Frank, J.L., Watkins, L.R., Maier, S.F., 2006. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. Journal of Neuroscience Methods 151, 121-130.

Godbout, J.P., Berg, B.M., Kelley, K.W., Johnson, R.W., 2004. alpha-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. Journal of Neuroimmunology 149, 101-109.

Godbout, J.P., Berg, G.M., Krzyszton, C., Johnson, R.W., 2005a. alpha-Tocopherol attenuates NF kappa B activation and pro-inflammatory cytokine production in brain and improves recovery from lipopolysaccharide-induced sickness behavior. Journal of Neuroimmunology 169, 97-105.

Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W., 2005b. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. FASEB Journal 19, 1329.

Godbout, J.P., Johnson, R.W., 2004. Interleukin-6 in the aging brain. Journal of Neuroimmunology 147, 141-144.

Godbout, J.P., Johnson, R.W., 2009. Age and neuroinflammation: A lifetime of psychoneuroimmune consequences. Immunology and Allergy Clinics of North America 29, 321.

Greenhill, C.J., Rose-John, S., Lissilaa, R., Ferlin, W., Ernst, M., Hertzog, P.J., Mansell, A., Jenkins, B.J., 2011. IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3. Journal of Immunology 186, 1199-1208.

Gruol, D.L., Nelson, T.E., 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Molecular Neurobiology 15, 307-339.

Henry, C.J., Huang, Y., Wynne, A., Hanke, M., Himler, J., Bailey, M.T., Sheridan, J.F., Godbout, J.P., 2008. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. Journal of Neuroinflammation 5.

Henry, C.J., Huang, Y., Wynne, A.M., Godbout, J.P., 2009. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1 beta and anti-inflammatory IL-10 cytokines. Brain Behavior and Immunity 23, 309-317.

Heyser, C.J., Masliah, E., Samimi, A., Campbell, I.L., Gold, L.H., 1997. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. Proceedings of the National Academy of Sciences of the United States of America 94, 1500-1505.

Hofmann, K.W., Schuh, A.F.S., Saute, J., Townsend, R., Fricke, D., Leke, R., Souza, D.O., Portela, L.V., Chaves, M.L.F., Rieder, C.R.M., 2009. Interleukin-6 serum levels in patients with Parkinson's disease. Neurochemical Research 34, 1401-1404.

Huang, Y., Henry, C.J., Dantzer, R., Johnson, R.W., Godbout, J.P., 2008. Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. Neurobiology of Aging 29, 1744-1753.

Jang, S., Dilger, R.N., Johnson, R.W., 2010. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. Journal of Nutrition 140, 140 (110) 1892-1898.

Jones, S.A., Richards, P.J., Scheller, J., Rose-John, S., 2005. IL-6 transsignaling: The in vivo consequences. Journal of Interferon and Cytokine Research 25, 241-253.

Jostock, T., Mullberg, J., Ozbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M.F., Rose-John, S., 2001. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. European Journal of Biochemistry 268, 160-167.

Kelley, K.W., Bluthe, R.M., Dantzer, R., Zhou, J.H., Shen, W.H., Johnson, R.W., Broussard, S.R., 2003. Cytokine-induced sickness behavior. Brain Behavior and Immunity 17, S112-S118.

Kishimoto, T., Akira, S., Taga, T., 1992. IL-6 receptor and mechanism of signal transduction International Journal of Immunopharmacology 14, 431-438.

Kozlova, E.N., Takenaga, K., 2005. A procedure for culturing astrocytes from white matter and the application of the siRNA technique for silencing the expression of their specific marker, S100A4. Brain Research. Brain research protocols 15, 59-65.

Krzyszton, C.P., Sparkman, N.L., Grant, R.W., Buchanan, J.B., Broussard, S.R., Woods, J., Johnson, R.W., 2008. Exacerbated fatigue and motor deficits in interleukin-10-deficient mice after peripheral immune stimulation. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 295, R1109-R1114.

Linker, R.A., Luhder, F., Kallen, K.J., Lee, D.H., Engelhardt, B., Rose-John, S., Gold, R., 2008. IL-6 transsignalling modulates the early effector phase of EAE and targets the blood-brain barrier. Journal of Neuroimmunology 205, 64-72.

Maggio, M., Guralnik, J.M., Longo, D.L., Ferrucci, L., 2006. Interleukin-6 in aging and chronic disease: A magnificent pathway. Journals of Gerontology Series 61, 575-584.

Matthews, V., Schuster, B., Schutze, S., Bussmeyer, I., Ludwig, A., Hundhausen, C., Sadowski, T., Saftig, P., Hartmann, D., Kallen, K.J., Rose-John, S., 2003. Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). Journal of Biological Chemistry 278, 38829-38839.

McLoughlin, R.M., Jenkins, B.J., Grail, D., Williams, A.S., Fielding, C.A., Parker, C.R., Ernst, M., Topley, N., Jones, S.A., 2005. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proceedings of the National Academy of Sciences of the United States of America 102, 9589-9594.

Mooradian, A.D., 1988. Effect of aging on the blood-brain barrier. Neurobiology of Aging 9, 31-39.

Morley, A.A., Trainor, K.J., 2001. Lack of an effect of vitamin E on lifespan of mice. Biogerontology 2, 109-112.

Nguyen, K., D'Mello, C., Le, T., Urbanski, S., Swain, M.G., 2011. Regulatory T cells suppress sickness behaviour development without altering liver injury in cholestatic mice. Journal of Hepatology.

Park, S.J., Lee, J.H., Kim, H.Y., Choi, Y.H., Park, J.S., Suh, Y.H., Park, S.M., Joe, E.H., Jou, I., 2012. Astrocytes, but not microglia, rapidly sense H2O2 via STAT6 phosphorylation, resulting in cyclooxygenase-2 expression and prostaglandin release. Journal of Immunology.

Perry, V.H., Newman, T.A., Cunningham, C., 2003. The impact of systemic infection on the progression of neurodegenerative disease. Nature Reviews Neuroscience 4, 103-112.

Quan, N., 2008. Immune-to-brain signaling: How important are the blood-brain barrier-independent pathways? Molecular Neurobiology 37, 142-152.

Raff, M.C., Fields, K.L., Hakomori, S.I., Mirsky, R., Pruss, R.M., Winter, J., 1979. Cell-type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. Brain Research 174, 283-308.

Richards, P.J., Nowell, M.A., Horiuchi, S., McLoughlin, R.M., Fielding, C.A., Grau, S., Yamamoto, N., Ehrmann, M., Rose-John, S., Williams, A.S., Topley, N., Jones, S.A., 2006. Functional characterization of a soluble gp130 isoform and its therapeutic capacity in an experimental model of inflammatory arthritis. Arthritis and Rheumatism 54, 1662-1672.

Richwine, A.F., Godbout, J.P., Berg, B.M., Chen, J., Escobar, J., Millard, D.K., Johnson, R.W., 2005. Improved psychomotor performance in aged mice fed diet high in antioxidants is associated with reduced ex vivo brain interleukin-6 production. Brain Behavior and Immunity 19, 512-520.

Rose-John, S., Heinrich, P.C., 1994. Soluble receptors for cytokines and growth factors: generation and biological function. Biochemical Journal 300, 281-290.

Rose-John, S., Waetzig, G.H., Scheller, J., Grotzinger, J., Seegert, D., 2007. The IL-6/sIL-6R complex as a novel target for therapeutic approaches. Expert Opinion on Therapeutic Targets 11, 613-624.

Rummel, C., Sachot, C., Poole, S., Luheshi, G.N., 2006. Circulating interleukin-6 induces fever through a STAT3-linked activation of COX-2 in the brain. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 291, R1316-R1326.

Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica Et Biophysica Acta-Molecular Cell Research 1813, 878-888.

Schobitz, B., Dekloet, E.R., Sutanto, W., Holsboer, F., 1993. Cellular localization of the interleukin-6 mRNA and interleukin-6 receptor mRNA in rat brain. European Journal of Neuroscience 5, 1426-1435.

Sparkman, N.L., Buchanan, J.B., Heyen, J.R.R., Chen, J., Beverly, J.L., Johnson, R.W., 2006. Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. Journal of Neuroscience 26, 10709-10716.

Sparkman, N.L., Johnson, R.W., 2008. Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. Neuroimmunomodulation 15, 323-330.

Tosato, G., Jones, K.D., 1990. Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. Blood 75, 1305-1310.

Weaver, J.D., Huang, M.H., Albert, M., Harris, T., Rowe, J.W., Seeman, T.E., 2002. Interleukin-6 and risk of cognitive decline - MacArthur studies of successful aging. Neurology 59, 371-378.

Ye, S.-M., Johnson, R.W., 1999a. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Ye, S.M., Johnson, R.W., 1999b. Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93, 139-148.

Ye, S.M., Johnson, R.W., 2001. Regulation of interleukin-6 gene expression in brain of aged mice by nuclear factor kappa B. Journal of Neuroimmunology 117, 87-96.

Chapter 7

Summary and Significance

The rapid growth of the aged population, along with healthcare reform and cost associated with supporting the aged, makes research on the pathogenesis of aging a vital concern to society. The U.S. census bureau projects that individuals aged 65 years and older will account for roughly 30% of the total population by 2050; up from about 10% in 2010 (U.S. Census). With this growing aging population, the prevalence of age-related diseases with an inflammatory component (e.g.arthritis, Alzheimer's disease, diabetes, cardiovascular disease) increases. Indeed, in the brain of aged animals, there is a baseline upregulation of pro-inflammatory cytokines, which are further exaggerated during peripheral infection when compared to young adult animals. Our lab and others have shown this exacerbated cytokine response in the brain of aged animals plays a critical role in infection-related behavioral pathology (Brod, 2000; Burton and Johnson, 2011; Burton, 2012 ; Burton et al., 2011; Chen et al., 2008; Gallagher et al., 2006; Gallagher et al., 1996; Godbout et al., 2005; Godbout et al., 2008).

In recent years, the numbers of publications centered on aging and neurodegeneration have steadily increased. With this increase, the focus on neuro-modulatory pathways has come into the forefront and glial cells and inflammatory processes in the brain are principal subjects of investigation. The central nervous system (CNS) had been typically regarded as an immunologically privileged site, though a plethora of studies have provided evidence that the peripheral immune system communicates an immune signal to the CNS via neural, humoral, and diffusive

pathways. This signal comes in the form of cytokines, which play a crucial role in the initiation, propagation, regulation, and suppression of immune and inflammatory processes. Pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 are the prototypic molecules that are of particular interest in CNS inflammation and pathogenesis of neurodegenerative disease.

There is a correlation with the over production IL-6 during aging and development of neurodegenerative diseases, such as Alzheimer's and Multiple Sclerosis (De Luigi et al., 2001; Gruol and Nelson, 1997; Licastro et al., 2003), and we believe the excessive production of IL-6 in the brain may underlie cognitive deficits that are highly prevalent in elderly patients. However IL-6 is implicated in pro- and anti-inflammatory processes. The data presented suggests that IL-6 *trans*-signaling is involved in the pro-inflammatory arm of IL-6 production in the CNS and that the use of sgp130 as an inhibitor of this pathway in an array of inflammatory conditions, from peripheral to neurodegenerative disease, mitigates IL-6 expression and has a beneficial effect on sickness-related behavioral and cognitive deficits.

7.1 Literature Cited

Brod, S.A., 2000. Unregulated inflammation shortens human functional longevity. Inflammation Research 49, 561 - 570.

Burton, M.D., Johnson, R.W., 2011. Interleukin-6 trans-signaling in the senescent mouse brain is involved in infection-related deficits in contextual fear conditioning. Brain Behavior and Immunity 26, 738-738.

Burton, M.D., Johnson, R.W., 2012 Interleukin-6 is involved in microglia priming and the exaggerated LPS-induced sickness response in aged mice. (in prep).

Burton, M.D., Sparkman, N.L., Johnson, R.W., 2011. Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. Journal of Neuroinflammation 8, 54.

Chen, J., Buchanan, J.B., Sparkman, N.L., Godbout, J.P., Freund, G.G., Johnson, R.W., 2008. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behavior and Immunity 22, 301-311.

De Luigi, A., Fragiacomo, C., Lucca, U., Quadri, P., Tettamanti, M., Grazia De Simoni, M., 2001. Inflammatory markers in Alzheimer's disease and multi-infarct dementia. Mech Ageing Dev 122, 1985-1995.

Gallagher, M., Colantuoni, C., Eichenbaum, H., Haberman, R.P., Rapp, P.R., Tanila, H., Wilson, I.A., 2006. Individual differences in neurocognitive aging of the medial temporal lobe. Age 28, 221-233.

Gallagher, M., Landfield, P.W., McEwen, B., Meaney, M.J., Rapp, P.R., Sapolsky, R., West, M.J., 1996. Hippocampal neurodegeneration in aging. Science 274, 484-485.

Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W., 2005. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. FASEB Journal 19, 1329.

Godbout, J.P., Moreau, M., Lestage, J., Chen, J., Sparkman, N.L., Connor, J.O., Castanon, N., Kelley, K.W., Dantzer, R., Johnson, R.W., 2008. Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system. Neuropsychopharmacology 33, 2341-2351.

Gruol, D.L., Nelson, T.E., 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Molecular Neurobiology 15, 307-339.

Licastro, F., Grimaldi, L.M., Bonafe, M., Martina, C., Olivieri, F., Cavallone, L., Giovanietti, S., Masliah, E., Franceschi, C., 2003. Interleukin-6 gene alleles affect the risk of Alzheimer's disease and levels of the cytokine in blood and brain. Neurobiolgy of Aging 24, 921-926.